Journal of Experimental Marine Biology and Ecology 442 (2013) 80–87

Contents lists available at SciVerse ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Insights on the demography of cryptic nesting by green turtles (*Chelonia mydas*) in the main Hawaiian Islands from genetic relatedness analysis

Amy Frey [a,*], Peter H. Dutton [a], George H. Balazs [b]

[a] Protected Resources Division, Southwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 8901 La Jolla Shores Dr., La Jolla, CA 92037, USA

[b] Pacific Islands Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2570 Dole Street, Honolulu, HI 96822, USA

ARTICLE INFO

Article history:
Received 22 June 2012
Received in revised form 6 December 2012
Accepted 30 January 2013
Available online xxxx

Keywords:
Chelonia mydas
Founder event
Kinship
Microsatellite
Relatedness
Sea turtles

ABSTRACT

Within the Hawaiian archipelago, green turtle nesting has occurred almost exclusively in the northwestern Hawaiian Islands, mainly at French Frigate Shoals (FFS), however an increase in occasional nesting has recently been observed on the main Hawaiian Islands (MHI). Due to logistical constraints, monitoring the nesting activity on the MHI has been limited to nest documentation. Without systematic tagging of the nesting females it is not clear how many are nesting here. We used mitochondrial (mt) DNA sequencing combined with nuclear (n) DNA analysis based on 14 microsatellite markers to infer the number of individual nesters. Genotypes were determined for 181 dead embryos and hatchlings salvaged from 71 nests laid on Maui, Molokai, Kauai, Lanai, and Oahu, along with those of 81 nesting females that were sampled on FFS. MtDNA results showed that 58% of the MHI clutches were laid by females with a relatively rare haplotype only reported in 16% of the FFS nesting population. Nuclear DNA results showed that nesting in the MHI might be attributed to a relatively small number of females that appear to be related to each other. We were able to reconstruct genotypes for nesting females from hatchling profiles and we estimate that 15 different females were responsible for clutches laid on the MHI. Taken together, the mtDNA and nDNA results suggest that the nesting population at the MHI may be the result of a few founders that originated from the FFS breeding population, possibly facilitated by captive rearing and release of FFS juveniles locally from Oahu. We suggest that this regional range expansion may buffer against the loss of current nesting sites at FFS due to sea level rise. Our results demonstrate the potential for genetic tools to be incorporated into population assessment, particularly in areas where access to reproductive females is difficult and population size is unknown.

Published by Elsevier B.V.

1. Introduction

Population assessments are essential for the conservation and management of endangered species. In order to assess and effectively manage species and populations, it is important to have a good understanding of key life history and demographic components (Wright et al., 2012) such as population size and trends, site fidelity, age to maturity and survival rates. Accurate population assessments are particularly challenging for migratory animals with complex life histories, such as marine turtles, where breeding, developmental and feeding habitats may be separated by thousand of kilometers, and direct observation of animals is often limited. For example, green turtles (*Chelonia mydas*) are listed on the IUCN Red List as Endangered (Seminoff, 2004), although several major populations have shown increasing trends for the last 35 years (Chaloupka et al., 2008), including Hawaii which is now listed as a population of Least Concern (Pilcher et al., 2012). As juveniles, green turtles spend their first 5–10 years in oceanic habitats before entering a more neritic subadult stage where they remain for about a decade or longer before maturity (Bolten, 2003). Once turtles are mature, males are rarely seen but females periodically haul out on beaches to lay clutches of eggs. A growing number of genetic studies have confirmed that these highly migratory marine animals return to their natal beach to nest (Bowen and Avise, 1996; Meylan et al., 1990). However, the level of philopatry may vary by species and geographic region, and some level of imprecision in natal homing is required for new nesting colonies to be established (Jensen et al., in press). In theory a new nesting colony might result from a founder whose offspring return later as adults to the new natal beach to nest, with the potential of genetic drift resulting in marked differentiation between nesting populations (Bowen and Avise, 1996). However, several sea turtle nesting colonies that have been extirpated over the last century remain extinct, and it is believed that recolonization is rare and occurs over evolutionary time scales (Bowen and Avise, 1996). Nevertheless, the tendency toward natal homing in sea turtles provided the basis for conservation efforts for the Critically Endangered Kemp's ridley (*Lepidochelys kempii*) in the Gulf of Mexico. This involved the relocation of eggs collected at the last remaining nesting site, Rancho

* Corresponding author. Fax: +1 858 546 7003.
E-mail address: Amy.Frey@noaa.gov (A. Frey).

0022-0981/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jembe.2013.01.030

Nuevo, Mexico to North Padre Island in Texas USA, where the resulting hatchlings were imprinted to the local beaches then either released or captive reared ("headstarted") in the laboratory at Galveston Texas for up to a year (Shaver, 2005). This approach has been costly and controversial, and has not been widely adopted as a conservation tool for sea turtles (Frazier, 1992). Although Kemp's ridley nesting has increased in recent years in Mexico and along the Texas coast, the impacts of headstarting remain unclear, and elimination of egg harvest and mortality in fisheries are believed to be the main reasons for the population recovery (Dutton et al., 2002). A similar situation exists at the aquarium at Sea Life Park on Oahu where several hundred captive-reared juvenile green turtles have been released since 1976. Sea Life Park (SLP) has had green turtles on display since it opened in the mid 1960s, and these animals, originally collected from FFS, serendipitously produced hatchlings in captivity. These hatchlings are reared in tanks for one year, and released as juveniles in local Hawaiian waters, however their subsequent survival and fate is unknown.

Marine turtle population assessments are often done on nesting beaches where there is access to nesting females. Counts of nesting females in combination with capture mark recapture (CMR) studies are generally used to estimate population abundance and demographic parameters (Balazs and Chaloupka, 2006; Chaloupka and Limpus, 2001). However, it is often not possible to monitor and tag nesters, and instead extrapolations based on nest or track counts are used to estimate population size (Chan, 2006; Troeng and Rankin, 2005). Without information on the annual number of clutches laid by each female and nesting periodicity, it is not possible to precisely determine the number of females in the population solely from nest counts. However, since mtDNA is inherited maternally (i.e., identical in mother and offspring) it is possible to survey the mtDNA variation among nesters by sequencing DNA obtained from one hatchling or embryo produced by each female. In the absence of parental identities, genetic markers may be used to clarify relationships among individuals and to establish reliable pedigrees; these may be useful for estimating population size (Blouin, 2003; Herbinger et al., 2006).

In Hawaii more than 90% of all green turtle nesting occurs at French Frigate Shoals (FFS) in the northwestern Hawaiian Islands. Mature females migrate approximately every three years from the foraging grounds, which are located predominantly around the main Hawaiian Islands (MHI) (Dutton et al., 2008), to the nesting beaches, where breeding takes place offshore (Niethammer et al., 1997). During the nesting season, females come ashore approximately every 13 days and lay 1–6 clutches (avg. = 1.8). Hatchlings emerge roughly 65 days later (Niethammer et al., 1997). The green turtles nesting on FFS have been monitored since 1973 and this population is one in the Pacific that has been steadily increasing in size (Balazs and Chaloupka, 2004, 2006; Chaloupka and Balazs, 2007).

In recent years sporadic nesting has been recorded on the MHI beaches (Fig. 1). Due to logistical constraints, monitoring nesting activity on the MHI has been mainly confined to daytime nest observation while the nesting females themselves are rarely observed. One of the few nesters that has been observed over the last decade is "turtle 5690", observed on Lahaina and identified by her original tag as a previous captive-held juvenile. She was collected along with 234 other hatchlings at FFS, and they were all held in captivity (at Sea Life Park on Oahu) for one year before being released off the MHI in 1981 (Balazs et al., 2004). The implication is that these captive-reared turtles, which may not have properly "imprinted" on the FFS beaches, selected a beach near the foraging habitat once they reached maturity and have demonstrated nest site fidelity as adults, however due to the cryptic nature of nesting in the MHI, it has not been possible to determine the number of females or identities of the nesters

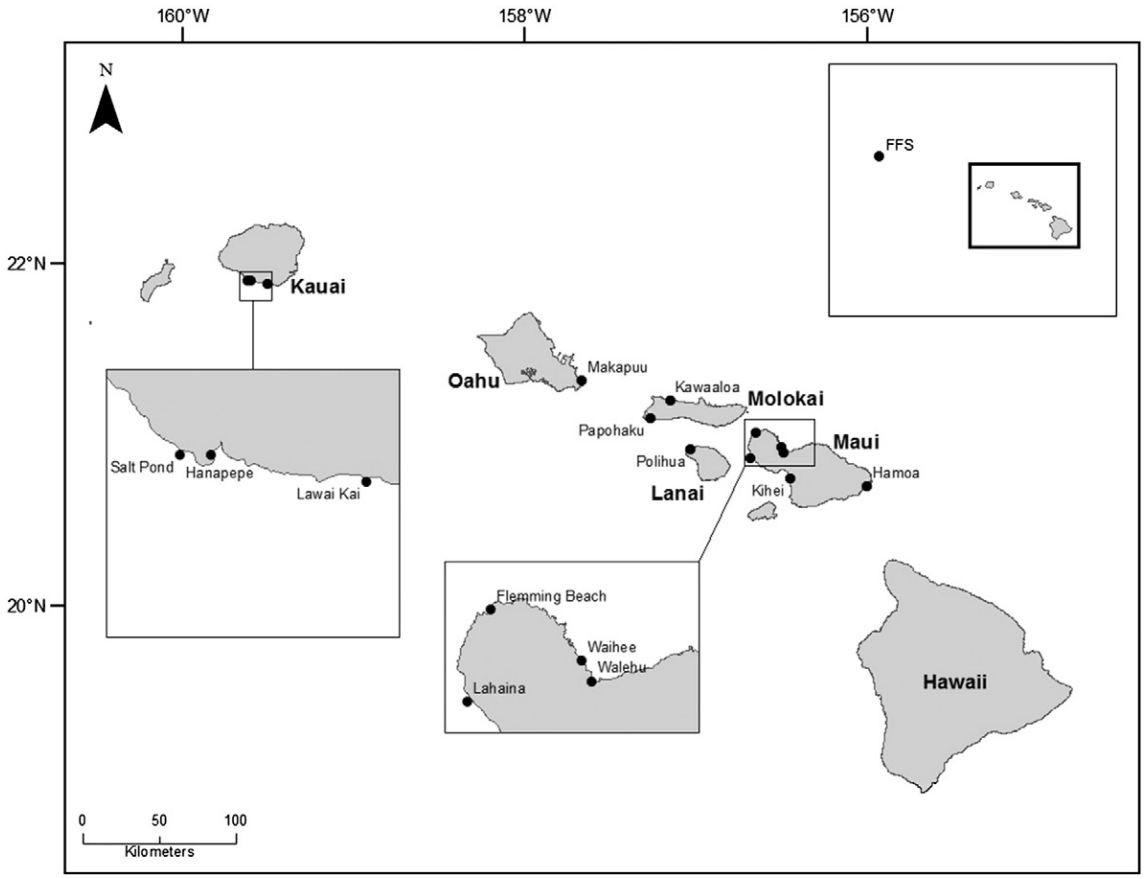

Fig. 1. Location of green turtle nesting sites sampled on the main Hawaiian Islands (MHI). Insert shows location of French Frigate Shoals (FFS) rookery in the northwestern archipelago.

responsible for the majority of nests and determine the demographic history and structure of this population. Nest locations and approximate deposition dates are routinely recorded. Nest contents are excavated and evaluated after the hatchlings have emerged. This practice offers an opportunity for salvaging tissue from dead embryos for genetic analysis. When the identities of the nesting females are unknown, genetic kinship approaches may be used to examine relatedness among clutches (offspring) and to determine which clutches were laid by the same unknown female. Furthermore, clutches may be matched to known females, and the offspring genotypes may be used to infer parental genotypes (Stewart and Dutton, 2011).

The purpose of this study was to determine how many females are responsible for MHI nests by evaluating the relatedness of the clutches in the absence of female nester samples and to characterize the genetic variation of the green turtles nesting on the MHI. This approach provides a novel tool to census small populations when traditional CMR techniques are not possible.

2. Materials and methods

2.1. Sample collection

Nest sampling on the MHI has been comprehensive, an average of 90% of the clutches laid each year since 2000 have been sampled. Once nests hatched, the contents were excavated and inventoried. Dead embryos and dead hatchling samples (n = 134) were salvaged from 55 clutches that were laid by unknown females on the MHI between 2000 and 2010. An additional 41 embryos or hatchlings were salvaged from 15 clutches (three in 2000, five in 2002, and seven in 2004) that were laid by a known female (Tag # 5690) on Lahaina beach on Maui (Balazs et al., 2004). Six embryos or hatchlings were also salvaged from a nest on Oahu where the female (Tag # Q795) was also sampled. There was an additional female sampled on Molokai. Nest sites are shown in Fig. 1. Samples from the FFS nesting females (n = 85) were collected during regular monitoring surveys from 1995 to 1997 (Dutton et al., 2008).

2.2. Laboratory analysis

We extracted genomic DNA from the female samples according to the methods described in Dutton et al. (2008), while DNA was extracted from the embryos and hatchlings using either the X-tractor Gene extraction robot (Corbett Robotics, San Francisco, CA, USA) or a salting out method (Miller et al., 1988). We genotyped each sample with 14 microsatellite loci: A6, B103, B108, B123, C102, D1, D2, D105, D108, and D115 following previously described protocols (Dutton and Frey, 2009). Four additional unpublished loci were used following the same protocols described by Dutton and Frey (2009): A1, B112, D102, and D107 (Genbank Accession numbers: EU668894, EU668896, EU668898, EU668899). PCR products were labeled using one of the standard sequencing dyes (HEX or FAM; Applied Biosystems, Inc., Foster City, CA, USA) and were separated on an ABI 3730 DNA analyzer with Genescan Rox500 fluorescent size standard (PE Applied Biosystems, Foster City, CA, USA). Additionally, we amplified and sequenced the mtDNA control region using primers described by LeRoux et al. (2012) for at least one sample from each of the 71 hatched nests from the MHI and all of the 81 FFS females sampled.

2.3. Statistical analysis

We aligned mtDNA sequences and assigned haplotypes using Seqscape v3.5 (PE Applied Biosystems, Foster City, CA, USA). Microsatellite data were analyzed using GeneMapper v4.0 (PE Applied Biosystems, Foster City, CA, USA). For quality control purposes, DNA was randomly selected from 10% of our samples and re-amplified and genotyped to obtain replicate products in order to quantify genotyping error rates. We tested all 14 markers for Mendelian inheritance using the known mother (Tag # 5690) and her 41 hatchlings from Lahaina, Maui. Standard population genetic parameters were computed for both the FFS population and the MHI females that were either sampled or identified using Colony v2.0, using both GENEPOP v4 (Raymond and Rousset, 1995) and MicroChecker v2 (Van Oosterhout et al., 2004).

To assess the relatedness of the 55 nests laid on the MHI for which the mother was not known, we used Colony v2.0 (Jones and Wang, 2010). Colony is used to determine full- and half-sibling relationships, and it allows sibship reconstruction without knowledge of the parental genotypes. In order to establish confidence in our analysis, we ran Colony iteratively five times, adjusting some of the parameters with each run in order to evaluate the consistency of the results (Table 2). For all runs, we allowed for both male and female polygamy and inbreeding (Lee, 2008) as input parameters, and chose the full likelihood model with either medium precision (runs 1–4) or high precision (run 5) as described in Wang and Santure (2009). For each run the allele frequency estimates were based on the hatchling data, and were calculated iteratively by Colony while searching for the maximum-likelihood configuration based on the inferred sibship and parentage relationships. The sibship size prior was left blank in each run. For the first run we included all 14 markers that were considered codominant, with the allelic dropout rate being zero; the error rate calculated during the genotyping replication exercise was used as an input variable. For the second run we only included the 10 markers that were in HWE. For the third, fourth, and fifth runs we included all 14 loci, and additionally used the genotyping error rates as the allelic dropout rate for four loci that were out of HWE and likely to exhibit large allelic dropout. In each of the analyses there were 181 offspring genotypes, zero candidate male genotypes and three candidate female genotypes. Two of the females were known to be mothers of some of the offspring; therefore the probability of an actual mother being included

Table 1
Mitochondrial DNA haplotypes for each nest by year and location. () represents the number of nests.

	Kauai	Lanai	Maui						Molokai		Oahu
			Lahaina	Flemming Beach	Hamoa	Kihei	Waihee	Walehu	Kawaaloa Bay	Papohaku	
2000			CmP2.1 (3)[a]			CmP1.1					
2001				CmP1.1							
2002			CmP2.1 (5)[a]				CmP3.2				
2003					CmP1.1						
2004			CmP2.1 (7)[a]				CmP3.2		CmP3.2 (2)		
2006									CmP3.2 (4)		CmP1.1[b]
2007	CmP1.1(6)							CmP3.2 (3)	CmP3.2 (10)		
2008		CmP2.1							CmP3.2 (3)	CmP2.1 (2)	CmP1.1 (2)
2009									CmP3.2 (9)		
2010									CmP3.2 (8)		

[a] Indicates nests laid by female with tag # 5690.
[b] Indicates nest laid by female with tag Q795.

Table 2
Colony runs. Parameters selected for each of the five iterations.

	Run 1	Run 2	Run 3	Run 4	Run 5
Precision of run	Med.	Med.	Med.	Med.	High
# of loci	14	10	14	14	14
Allelic dropout rate included	No	No	Yes	No	Yes
Known maternal sibship included	Yes	Yes	Yes	No	Yes
Excluded females	Yes	Yes	Yes	No	Yes
Excluded maternal sibships	Yes	Yes	Yes	No	Yes

within the candidate genotypes was one. Because none of the fathers had been previously identified, the known paternal sibship was zero, and any samples known to be from the same nest were coded as a known maternal sibship (except in our fourth run where we didn't provide Colony with any prior information).

For each run, except the fourth, we excluded any female that didn't have the same maternal mtDNA haplotype as the hatchling set. Additionally, there were certain conditions that required some hatchling sets (nests) to be excluded as potential siblings from other hatchling sets (nests). Those conditions included any hatchling sets that had different mtDNA haplotypes, and therefore could not have been siblings. It was likewise not possible for nests (hatchling sets) with oviposition dates less than eight days apart to be maternal siblings because the range for the internesting interval of Hawaiian green turtles is 11–18 days (Niethammer et al., 1997). Any hatchling samples from nests that were laid in consecutive years were also excluded as potential maternal siblings because it is unlikely that a female would nest in consecutive years (Bjorndal, 1997). Pairwise comparisons were generated for each hatchling, and the most likely relationship between a pair of hatchlings was calculated. The most likely parents (mother and father) were assigned to each hatchling. For each parent, possible genotypic fingerprints were generated along with the probability that the genotype for each marker was correct. We used the relationships assigned in Colony to group the nests into known-mother sets and then compared the relationships manually. Putative maternal genotypes were assigned by Colony for each hatchling set.

We used Arlequin v3.5 (Excoffier and Lischer, 2010) to test for significant differences between the mtDNA haplotype frequencies of nesting females from FFS and those estimated for the MHI.

3. Results

Three haplotypes that are commonly found at FFS were also found in the nests on the MHI (Table 1). Of the 38 clutches laid on Molokai from 2004 to 2010, 36 of them were laid at Kawaaloa Bay and shared the same haplotype (CmP3.2). The other two clutches on Molokai (from Papohaku), had a different haplotype (CmP2.1).

All samples were genotyped at 14 loci, and the summary statistics for each locus, including the probability of identity and genotyping error rates for each marker are shown in Table 3. MicroChecker results showed that four markers were out of HWE in the FFS population with possible large allelic dropout present.

The results from each of the five Colony runs may be found in the Supplementary material (Tables S1, S2). Here we describe the results from the fifth colony run. We were able to 1) assign the 16 nests from two known females to their correct mothers, 2) reconstruct the female genotypes with confidence (Table 4) and 3) assign the 55 clutches laid by unknown mothers to an estimated 15 females, including the two known nesters that were sampled.

Of 71 clutches, there were 41 with the maternal haplotype CmP3.2, which were assigned to eight different females; seven of those females returned to nest in multiple years (Table 5). Five clutches were laid on Maui between 2002 and 2007 and were assigned to Female 1. Thirty-six of the clutches that were laid on the same beach on Molokai between 2004 and 2010 were assigned to Females 2 through 8.

Table 3
Summary statistics for 14 loci used to genotype hatchling and mother turtles. We report the number of females (N), the number of alleles, the observed heterozygosity (Ho), the Hardy–Weinberg p-value (Phw), the probability of identity (PID) and the error rate.

Locus	N	N alleles	Ho	Phw	PID	Error rate
A1	84	7	0.714	0.935	0.374	0.0125
A6	88	8	0.727	0.369	0.402	0.0326
B103	92	5	0.615	0.457	0.534	0.0001
B108	74	5	0.716	0.257	0.425	0.0405
B112	84	12	0.881	0.814	0.157	0.0131
B116	74	3	0.311	0.014	0.73	0.0149
B123	86	3	0.337	0.452	0.778	0.0001
C102	92	4	0.663	0.022	0.464	0.0235
D1	90	13	0.611	0.023	0.19	0.0001
D102	91	3	0.593	0.119	0.75	0.0001
D105	76	13	0.75	0.076	0.204	0.0042
D108	88	8	0.682	0.013	0.223	0.0112
D115	94	15	0.862	0.335	0.176	0.0195
D2	81	11	0.716	0.664	0.252	0.0001

There were 18 clutches with the maternal haplotype CmP2.1 that were assigned to two different females (Table 5). One of the females, Female 15 (Tag # 5690) was seen and sampled while she was laying the clutches at Lahaina, Maui between 2000 and 2004. The unknown clutch laid in 2008 on Lanai, which is about 30 km away from Lahaina, was also assigned to Female 15. There were also two unknown clutches laid at Papohaku (Molokai) in 2008 that were assigned to Female 9.

There were 12 clutches laid with the maternal haplotype CmP1.1 by an estimated five females (Table 5). One of the known females, Female 14 (Tag # Q795) laid one clutch on Oahu in 2006 and was assigned to another in 2008. Female 10 was assigned to all six of the unknown clutches in 2007 on Kauai. Female 11 was assigned to two clutches on Maui, one in 2000 and one in 2003. Female 12 was assigned to a clutch in 2008 on Oahu. Female 13 was assigned to a clutch on Maui in 2001.

For the FFS samples ($n=81$), CmP1.1 was most common ($n=49$) relative to CmP2.1 ($n=10$), CmP3.2 ($n=12$), and an additional haplotype, CmP3.1 ($n=10$) found in the FFS nesting population. A comparison of these haplotype frequencies indicated significant differentiation between FFS and MHI ($F_{ST}=0.25$, $p<0.0001$).

4. Discussion

Based on our combined mtDNA and microsatellite analysis results, we conclude that there are an estimated 15 females nesting on the MHI, and that they comprise a breeding population that is demographically distinct from the FFS nesting population. The significant genetic differentiation we found in the haplotype frequencies ($F_{ST}=0.25$, $p<0.0001$) between the MHI and FFS nesting populations suggests that the nests on the MHI are not the result of nesting by females that have "switched" nesting sites from FFS, but rather that the MHI nesting population was founded by individuals that colonized these new nesting sites. Interestingly, the haplotype CmP3.2, which is most commonly found among the MHI nests, is not the common FFS haplotype. At FFS, CmP3.2 is present in only 15% of the nesting females. This proportion is based on the extensive sampling of FFS nesting turtles as well as turtles in foraging populations and from strandings throughout the archipelago (Dutton et al., 2008). Haplotype CmP2.1 is also a relatively rare haplotype at FFS, and CmP3.1 was only found at FFS. Given the small size of the MHI nesting population, the significant haplotype frequency shift that we observed is likely the result of genetic drift caused by a founder event. Since three of the four FFS haplotypes are represented in the MHI, we conclude that the MHI population was founded by a minimum of three turtles. The lack of any new haplotypes in the MHI population suggests that the population was established relatively recently, since insufficient time has elapsed to accumulate new mutations. There is no evidence

Table 4
Genotype assignments made by the software program Colony for each female and locus. The probability of each genotype being correct is below the genotype, and the average probability is also included.

Female	A6		D1		D2		D108		A1		B103		B108		B112		B116		B123		C102		D102		D105		D115		Avg. prob.
#1	129	133	221	233	300	304	245	245	205	205	160	160	189	192	237	237	235	238	217	217	233	233	154	162	172	172	164	200	
	0.88		0.55		1.00		0.96		0.96		1.00		1.00		1.00		0.94		1.00		1.00		0.65		0.99		1.00		0.92
#2	129	131	229	269	304	304	241	245	191	203	160	168	186	189	237	249	235	238	217	217	235	237	154	162	160	164	164	196	
	1.00		1.00		1.00		1.00		1.00		1.00		0.99		1.00		0.97		1.00		1.00		1.00		1.00		1.00		1.00
#3	129	131	221	229	284	304	237	241	203	205	160	160	186	189	243	255	235	238	217	217	235	235	162	162	144	160	164	200	
	0.68		1.00		1.00		1.00		1.00		0.81		1.00		1.00		1.00		1.00		0.76		1.00		1.00		0.84		0.93
#4	129	131	221	229	284	324	237	241	203	205	160	160	186	192	237	255	235	238	217	217	237	237	154	162	144	172	164	200	
	1.00		1.00		0.97		0.46		1.00		0.92		0.99		0.51		0.52		1.00		0.89		0.56		0.45		0.97		0.80
#5	131	131	227	229	284	304	237	241	203	203	160	160	186	192	237	243	235	235	217	217	235	237	162	162	144	160	164	164	
	0.53		1.00		1.00		1.00		1.00		1.00		1.00		0.85		1.00		0.97		0.95		1.00		1.00		0.97		0.95
#6	129	131	229	233	300	304	237	249	195	205	160	160	186	189	237	255	235	235	217	220	235	235	154	162	144	160	164	164	
	0.91		1.00		1.00		0.99		0.96		0.98		0.94		0.68		0.71		1.00		0.86		1.00		0.97		0.87		0.92
#7	127	129	229	235	284	284	237	245	203	207	160	163	186	192	237	243	235	238	217	217	235	237	154	154	148	160	164	164	
	0.78		1.00		0.76		0.99		0.50		0.99		0.96		0.62		0.77		1.00		0.81		0.82		0.64		0.77		0.81
#8	127	129	229	237	284	308	241	245	191	191	160	160	186	189	243	249	235	238	217	217	235	237	154	154	148	164	196	200	
	0.82		1.00		1.00		0.99		0.90		1.00		0.91		0.99		1.00		1.00		0.92		0.94		1.00		0.93		0.96
#9	127	131	221	229	300	300	245	257	203	203	160	160	189	192	231	237	238	238	217	223	237	241	162	162	144	176	164	164	
	0.53		0.50		0.96		0.71		0.76		0.95		0.53		0.89		0.71		0.95		0.96		0.94		0.99		0.70		0.79
#10	127	129	221	227	300	304	249	253	203	205	160	175	189	189	243	246	238	238	217	217	235	235	162	162	140	176	164	184	
	0.72		0.72		1.00		1.00		0.93		1.00		0.81		0.91		0.76		0.99		0.84		0.97		1.00		1.00		0.90
#11	129	131	221	233	288	304	241	253	205	205	160	168	186	189	254	255	235	238	217	217	233	241	154	162	136	136	152	156	
	0.99		1.00		1.00		1.00		0.77		0.96		0.78		0.99		0.98		1.00		0.88		0.63		0.90		0.73		0.90
#12	131	131	221	233	304	320	241	241	199	203	160	160	186	189	237	243	235	238	217	220	235	235	154	162	144	148	164	200	
	0.42		0.50		0.50		0.29		0.32		0.77		0.33		0.49		0.47		0.75		0.66		1.00		0.50		0.48		0.53
#13	129	131	203	227	316	328	241	253	203	203	166	168	189	189	234	249	232	235	217	217	237	241	154	162	144	160	192	192	
	0.99		0.93		0.75		0.96		0.54		1.00		0.51		1.00		0.96		1.00		0.90		1.00		1.00		0.90		0.89

Table 5
Nest assignments for the 71 nests sampled on the main Hawaiian Islands including a nest #, nest location, maternal haplotype, date, and the number of hatchlings sampled from each nest.

	Nest #	Location	Haplotype	Date	# of hatchlings
Female 1					
	1	Maui, Waihee	CmP3.2	9/18/02	4
	2	Maui, Waihee	CmP3.2	6/30/04	3
	3	Maui, Walehu	CmP3.2	6/6/07	2
	4	Maui, Walehu	CmP3.2	6/24/07	4
	5	Maui, Walehu	CmP3.2	7/9/07	4
Female 2					
	6	Molokai, Kawaaloa	CmP3.2	2004	1
	15	Molokai, Kawaaloa	CmP3.2	7/24/07	1
	16	Molokai, Kawaaloa	CmP3.2	8/19/07	1
	17	Molokai, Kawaaloa	CmP3.2	9/13/07	2
	18	Molokai, Kawaaloa	CmP3.2	8/5/07	1
	19	Molokai, Kawaaloa	CmP3.2	8/29/07	1
	20	Molokai, Kawaaloa	CmP3.2	10/19/07	2
	34	Molokai, Kawaaloa	CmP3.2	7/25/10	1
	38	Molokai, Kawaaloa	CmP3.2	9/15/10	4
	39	Molokai, Kawaaloa	CmP3.2	10/13/10	4
Female 3					
	7	Molokai, Kawaaloa	CmP3.2	2004	1
	8	Molokai, Kawaaloa	CmP3.2	8/8/06	2
	9	Molokai, Kawaaloa	CmP3.2	9/15/06	2
	10	Molokai, Kawaaloa	CmP3.2	11/9/06	1
	11	Molokai, Kawaaloa	CmP3.2	2006	2
	25	Molokai, Kawaaloa	CmP3.2	7/30/09	1
	26	Molokai, Kawaaloa	CmP3.2	8/13/09	1
	31	Molokai, Kawaaloa	CmP3.2	9/4/09	3
Female 4					
	12	Molokai, Kawaaloa	CmP3.2	7/28/08	2
	14	Molokai, Kawaaloa	CmP3.2	11/4/08	3
	36	Molokai, Kawaaloa	CmP3.2	8/25/10	1
Female 5					
	13	Molokai, Kawaaloa	CmP3.2	7/31/08	1
	35	Molokai, Kawaaloa	CmP3.2	8/6/10	1
	37	Molokai, Kawaaloa	CmP3.2	9/2/10	3
	40	Molokai, Kawaaloa	CmP3.2	10/15/10	2
	41	Molokai, Kawaaloa	CmP3.2	11/21/10	2
Female 6					
	21	Molokai, Kawaaloa	CmP3.2	8/12/07	1
	24	Molokai, Kawaaloa	CmP3.2	11/2/07	3
	27	Molokai, Kawaaloa	CmP3.2	7/30/09	1
	28	Molokai, Kawaaloa	CmP3.2	9/9/09	1
Female 7					
	22	Molokai, Kawaaloa	CmP3.2	8/20/07	2
	23	Molokai, Kawaaloa	CmP3.2	9/5/07	1
	30	Molokai, Kawaaloa	CmP3.2	8/29/09	4
Female 8					
	29	Molokai, Kawaaloa	CmP3.2	8/13/09	4
	32	Molokai, Kawaaloa	CmP3.2	9/6/09	3
	33	Molokai, Kawaaloa	CmP3.2	2009	3
Female 9					
	42	Molokai, Papohaku	CmP2.1	9/22/08	3
	43	Molokai, Papohaku	CmP2.1	10/5/08	3
Female 15 (Tag # 5690)					
	44	Maui, Lahaina[a]	CmP2.1	10/16/00	2
	45	Maui, Lahaina[a]	CmP2.1		2
	46	Maui, Lahaina[a]	CmP2.1	11/15/00	2
	47	Maui, Lahaina[a]	CmP2.1	7/24/02	4
	48	Maui, Lahaina[a]	CmP2.1	8/6/02	4
	49	Maui, Lahaina[a]	CmP2.1	8/24/02	1
	50	Maui, Lahaina[a]	CmP2.1	10/4/02	1
	51	Maui, Lahaina[a]	CmP2.1	10/16/02	2
	52	Maui, Lahaina[a]	CmP2.1	5/7/04	1
	53	Maui, Lahaina[a]	CmP2.1	5/24/04	4
	54	Maui, Lahaina[a]	CmP2.1	6/7/04	4
	55	Maui, Lahaina[a]	CmP2.1	6/21/04	4
	56	Maui, Lahaina[a]	CmP2.1	7/6/04	4
	57	Maui, Lahaina[a]	CmP2.1	7/20/04	2
	58	Maui, Lahaina[a]	CmP2.1	8/3/04	4
	59	Lanai	CmP2.1	8/22/08	5
Female 10					
	63	Kauai	CmP1.1	7/4/07	1
	64	Kauai	CmP1.1	2007	5
	65	Kauai	CmP1.1	2007	4
	66	Kauai	CmP1.1	2007	3
	67	Kauai	CmP1.1	2007	3
	68	Kauai	CmP1.1	11/16/07	4
Female 11					
	61	Maui, Kihei	CmP1.1	9/27/00	2
	62	Maui, Hamoa	CmP1.1	6/12/03	4
Female 12					
	71	Oahu, Makapuu	CmP1.1	7/26/08	2
Female 13					
	60	Maui, Flemming Beach	CmP1.1	2001	8
Female 14 (Tag # Q795)					
	69	Oahu, MakaPuu[a]	CmP1.1	8/21/06	6
	70	Oahu, Makapuu	CmP1.1	8/4/08	1

[a] Indicates the nest was laid by a known female.

that the nesters themselves are switching from FFS as adults to nest instead on the MHI beaches.

Evaluation of the genotypic data and the relatedness of the nests to one another provided the opportunity to group the nests, reconstruct maternal genotypes, and estimate the number of breeding females. Having multiple offspring from the same nest, and prior knowledge of natal homing provides an advantage in parental reconstruction analysis (Jones et al., 2010). For approximately 55% of the nests sampled in this study, we only had one or two hatchling samples. In a population of unrelated individuals, such data may have tested the limitations of the Colony analysis. Wang and Santure (2009) show that the accuracy of parentage inference, in terms of correct assignment is around 30% with only one offspring sampled and about 55% with two sampled. However, we were able to establish confidence in the analysis by performing five iterations as described in the methods. For runs 1, 3, and 5, Colony assigned 15 females to the 71 nests. Some of the individual nest assignments grouped differently, or "floated" during the different runs; this uncertainty is likely due to the close relationship of the females to each other. Nest assignments were more robust in the cases where there were multiple hatchling samples from a nest and multiple clutches laid by the same female in subsequent years. These findings confirm that the sporadic nesting on the MHI may be traced to a small group of related females and that it is likely the result of a few founders.

In addition, parental genotype reconstruction analysis is unlikely to be affected by low levels of genotyping error because the error often affects only one or a few offspring at a single locus, and the unexpected genotypes of the affected offspring may be accounted for (Jones and Ardren, 2003). For one of the iterations (run 2), 10 loci were analyzed instead of 14 because we removed the loci that were out of HWE and were thus suspected of exhibiting allelic dropout. In this run, Colony assigned 14 females, ultimately assigning one less female to the 36 nests from Kawaaloa, Molokai. When we evaluated these results manually we found that some of the assignments were not biologically possible. Because Colony does factor in allelic dropout rates as well as genotyping error rates, we included the four loci that were out of HWE in our final analysis. Our genotyping error ranged from 0.0405 to 0.0001 with the average being 0.0123 and although this rate is very low, it is likely a result of the low quality/quantity of template DNA that is typical of studies employing non-invasive tissue sampling (Hoffman and Amos, 2005), such as salvaged nest contents. Stewart and Dutton (2011) reported a lower genotyping error rate (0.002) for samples from live hatchlings.

Genetic identification of female nesters, either directly or by genotype reconstruction from hatchling profiles, as we have done here, not only provides a tool ("genetic tag") for CMR studies (Stewart and Dutton, 2011) but also allows for further evaluation of relatedness

among nesting females, while allowing for the exploration of connectivity and evolutionary relationships at the population level. Our results suggest that the MHI nesting population is likely to be demographically isolated because it originated from a few founders.

One possible origin of the MHI founders is the aquarium at Sea Life Park on Oahu where several hundred captive-reared juvenile green turtles have been released since 1976. It is possible that the routine release of captive-reared yearling turtles has facilitated this colonization, or alternatively that this is also a natural consequence of the steady growth in numbers of the FFS nesting population over the last 35 years (Chaloupka et al., 2008). Our findings that the putative MHI nesters are closely related to turtle "5690" who was one of an early batch of juveniles held and released from SLP suggest that some of the other cryptic nesters may include others that may have originally been collected at FFS possibly from the same clutch. Interpretation of these results is difficult, since there are no records available of their history, and further confounded since they were not from clutches hatched at SLP, but were collected from the wild as hatchlings from FFS. Genotyping the breeding adults at SLP and all the juveniles that are released in the future would allow direct evaluation of the extent to which the captive reared animals recruit into the MHI nesting population.

The nesting population at FFS is one of the few populations of green turtles in the Pacific that has been increasing over the last 35 years (Balazs and Chaloupka, 2006; Tiwari et al., 2010). Since nesting is restricted to such a small geographic area, there is concern that the Hawaiian population may be vulnerable to the effects of sea level rise that may inundate the FFS nesting habitat (Baker et al., 2006; Tiwari et al., 2010). Regardless of the source of the founders, our findings suggest that it is possible for new rookeries to become established on the main islands and that this colonization has resulted in the potential for further increases in MHI nesting as hatchlings produced recently on these beaches mature and begin to nest in the future. This work provides an opportunity to gain insights into how new rookeries are established, and may represent the first documentation of genetic drift as it is occurring.

This study illustrates the utility of genetic tools for population assessment using offspring and their relatedness; this has not been done previously with marine turtles. Continued monitoring of the MHI population, and an expansion of the sampling effort of the nesters will provide a valuable opportunity to gain insights into the processes involved in colonization of new nesting habitat, the potential influence of captive rearing and release programs, and how founder events and genetic drift influence patterns of genetic variation in marine vertebrates.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2013.01.030.

Acknowledgments

Thanks to the Molokai Turtle Trackers, Nature Conservancy community volunteers who monitored nesting tracks and nests. Samples from the Hawaiian Islands National Wildlife Refuge were overseen by the USFWS, Department of the Interior. We'd like to thank Stacy Hargrove, Kelly Stewart, Erin LaCasella, Amy Jue, Gabriela Serra-Valente, Amanda Bowman, Michael Jensen, Suzanne Roden, Brad McDonald and Robin LeRoux for their help at the lab and with the data. This study was funded by NOAA-National Marine Fisheries Service. [**RH**]

References

Baker, J., Littnan, C., Johnston, D., 2006. Potential effects of sea level rise on the terrestrial habitats of endangered and endemic megafauna in the Northwest Hawaiian Islands. Endanger. Species Res. 2, 21–30.

Balazs, G.H., Chaloupka, M., 2004. Thirty-year recovery trend in the once depleted Hawaiian green sea turtle stock. Biol. Conserv. 117, 491–498.

Balazs, G.H., Chaloupka, M., 2006. Recovery trend over 32 years at the Hawaiian green turtle rookery of French Frigate Shoals. Atoll Res. Bull. 543, 147–158.

Balazs, G.H., Nakai, G.L., Hau, S., Grady, M.J., Gilmartin, W.G., 2004. Year 2000 Nesting of a captive-reared Hawaiian green turtle tagged and released as a yearling. In: Coyne, Michael, Clark, R. (Eds.), Proceedings of the 21st Annual Symposium on Sea Turtle Biology and Conservation. NOAA Tech. Memo. NMFS-SEFSC-528, Springfield, VA, pp. 100–101.

Bjorndal, K.A., 1997. Foraging ecology and nutrition of sea turtles. In: Lutz, P.L., Musick, J.A. (Eds.), The Biology of Sea Turtles, volume 1. CRC Press, Boca Raton, FL., pp. 199–222.

Blouin, M., 2003. DNA-based methods for pedigree reconstruction and kinship analysis in natural populations. Trends Ecol. Evol. 18, 503–511.

Bolten, A.B., 2003. Variation in sea turtle life history patterns: neritic vs. oceanic developmental stages. In: Lutz, P.L., Musick, J.A., Wyneken, J. (Eds.), The Biology of Sea Turtles, volume II. CRC Press, Boca Raton, FL, pp. 243–257.

Bowen, B.W., Avise, J.C., 1996. Conservation genetics of marine turtles. In: Avise, J.C., Hamrick, J.L. (Eds.), Conservation Genetics: Case Histories from Nature. Chapman & Hall, New York, pp. 190–237.

Chaloupka, M., Balazs, G.H., 2007. Using Bayesian state-space modelling to assess the recovery and harvest potential of the Hawaiian green sea turtle stock. Ecol. Model. 205, 93–109.

Chaloupka, M., Limpus, C.J., 2001. Trends in the abundance of sea turtles resident in Southern Great Barrier Reef waters. Biol. Conserv. 102, 235–249.

Chaloupka, M., Bjorndal, K.A., Balazs, G.H., Bolten, A.B., Ehrhart, L.M., Limpus, C.J., Suganuma, H., Troëng, S., Yamaguchi, M., 2008. Encouraging outlook for recovery of a once severely exploited marine megaherbivore. Glob. Ecol. Biogeogr. 17, 297–304.

Chan, E.H., 2006. Marine turtles in Malaysia: on the verge of extinction? Aquat. Ecosyst. Health Manag. 9, 175–184.

Dutton, P.H., Frey, A., 2009. Characterization of polymorphic microsatellite markers for the green turtle (*Chelonia mydas*). Mol. Ecol. Resour. 9, 354–356.

Dutton, P.H., Sarti, L., Márquez, R., Squires, D., 2002. Sea turtle conservation across the shared marine border. In: Fernandez, L., Carson, R.T. (Eds.), Both Sides of the Border: Transboundary Environmental Management Issues Facing Mexico and the United States. Kluwer Academic Publishers, pp. 429–454.

Dutton, P.H., Balazs, G.H., LeRoux, R.A., Murakawa, S.K.K., Zarate, P., Martínez, L.S., 2008. Composition of Hawaiian green turtle foraging aggregations: mtDNA evidence for a distinct regional population. Endanger. Species Res. 5, 37–44.

Excoffier, L., Lischer, H.E.L., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10, 564–567.

Frazier, N., 1992. Sea turtle conservation and halfway technology. Conserv. Biol. 6, 179–184.

Herbinger, C.M., O'reilly, P.T., Verspoor, E., 2006. Unravelling first-generation pedigrees in wild endangered salmon populations using molecular genetic markers. Mol. Ecol. 15, 2261–2275.

Hoffman, J.I., Amos, W., 2005. Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. Mol. Ecol. 14, 599–612.

Jensen, M.P., FitzSimmons, N.N., Dutton, P.H., 2013. Molecular Genetics of Sea Turtles. In: Wyneken, J., Lohman, K.J., Musick, J.A. (Eds.), The Biology of Sea Turtles, Volume III. CRC Press, Boca Raton, FL, pp. 135–154.

Jones, A.G., Ardren, W.R., 2003. Methods of parentage analysis in natural populations. Mol. Ecol. 12, 2511–2523.

Jones, O.R., Wang, J., 2010. COLONY: a program for parentage and sibship inference from multilocus genotype data. Mol. Ecol. Resour. 10, 551–555.

Jones, A.G., Small, C.M., Paczolt, K.A., Ratterman, N.L., 2010. A practical guide to methods of parentage analysis. Mol. Ecol. Resour. 10, 6–30.

Lee, P.L.M., 2008. Molecular ecology of marine turtles: new approaches and future directions. J. Exp. Mar. Biol. Ecol. 356, 25–42.

LeRoux, R.A., Dutton, P.H., Abreu-Grobois, F.A., Lagueux, C.J., Campbell, C.L., Delcroix, E., Chevalier, J., Horrocks, J.A., Hillis-Starr, Z., Troëng, S., Harrison, E., Stapleton, S., 2012. Re-examination of population structure and phylogeography of hawksbill turtles in the wider Caribbean using longer mtDNA sequences. J. Hered. 103, 806–820.

Meylan, A.B., Bowen, B.W., Avise, J.C., 1990. A genetic test of the natal homing versus social facilitation models for green turtle migration. Science 248, 724–726.

Miller, S.A., Dykes, D.D., Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215.

Niethammer, K.R., Balazs, G.H., Hatfield, J.S., Nakai, G.L., Megyesu, J.L., 1997. Reproductive biology of the green turtle (*Chelonia mydas*) at Tern Island, French Frigate Shoals, Hawai'i. Pac. Sci. 51, 36–47.

Pilcher, N.J., Chaloupka, M.Y., Woods, E., 2012. Chelonia mydas (Hawaiian subpopulation). In: IUCN (Ed.), IUCN Red List of Threatened Species. Version 2012.2. <www.iucnredlist.org>. Downloaded on 25 February 2013.

Raymond, M., Rousset, F., 1995. An exact test for population differentiation. Evolution 49, 1280–1283.

Seminoff, J.A. (Southwest Fisheries Science Center, U.S.) 2004. *Chelonia mydas*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. <www.iucnredlist.org>. Downloaded on 25 February 2013.

Shaver, D.J., 2005. Analysis of the Kemp's Ridley Imprinting and Headstart Project at Padre Island National Seashore, Texas, 1978–88, with subsequent nesting and stranding records on the Texas coast. Chelonian Conserv. Biol. 4, 846–859.

Stewart, K.R., Dutton, P.H., 2011. Paternal genotype reconstruction reveals multiple paternity and sex ratios in a breeding population of leatherback turtles (*Dermochelys coriacea*). Conserv. Genet. 12, 1101–1113.

Tiwari, M., Balazs, G.H., Hargrove, S., 2010. Estimating carrying capacity at the green turtle nesting beach of East Island, French Frigate Shoals. Mar. Ecol. Prog. Ser. 419, 289–294.

Troeng, S., Rankin, E., 2005. Long-term conservation efforts contribute to positive green turtle *Chelonia mydas* nesting trend at Tortuguero, Costa Rica. Biol. Conserv. 121, 111–116.
Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M., Shipley, P., 2004. Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes 4, 535–538.
Wang, J., Santure, A.W., 2009. Parentage and sibship inference from multilocus genotype data under polygamy. Genetics 181, 1579–1594.
Wright, L.I., Fuller, W.J., Godley, B.J., McGowen, A., Tregenza, T., Broderick, A.C., 2012. Reconstitution of paternal genotypes over multiple breeding seasons reveals male green turtles do not breed annually. Mol. Ecol. 21, 3626–3635.