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Genetic Sourcing for the Hawksbill Turtle, *Eretmochelys imbricata*, in the Northern Caribbean Region

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ABSTRACT. – **The mitochondrial control region of the hawksbill turtle, *Eretmochelys imbricata*, was analyzed using 70 nesting and 218 foraging samples from Cuba, 53 nesting and 21 foraging samples from Mexico, and 20 nesting and 106 foraging samples from Puerto Rico. From the 488 samples, 28 polymorphic sites defining 28 haplotypes were observed in 480 bp fragments. The most common haplotypes in the Cuban, Mexican, and Puerto Rican nesting populations were CU1, MXl, and PR1, respectively, showing that the nesting populations had the specific set of haplotypes as genetic markers. The one exception was PRl, the main haplotype for the Puerto Rican nesting population, which was also detected in one individual from the Cuban nesting population. Contribution rate for the Cuban foraging samples from the main southeast nesting area was the highest (70%), decreasing at the southwest (46%), and at the northeast (42%). Mexican foraging samples also had a high conribution rate of the local nesting haplotypes (71%), and the Puerto Rican nesting haploypes were moderately represented in their foraging samples (41%).**

KEY WORDS. – **Reptilia; Testudines; Cheloniidae; *Eretmochelys imbricata*; sea turtle; genetics; genetic sourcing; mitochondrial control region; natal homing; nesting population; foraging population; Cuba; Puerto Rico; Mexico; Caribbean Sea**

The molecular evolution of marine turtles has been well-studied in recent years. Avise et al. (1992) and Bowen et al. (1993) studied restriction-site (RFLP) and nucleotide sequence analyses for mitochondrial DNA (mtDNA) of marine turtles at intra- and interspecific levels, and showed that the cytochrome-b region of mtDNA evolution in turtles proceeds at only 10-20% of the conventional vertebrate pace. Nuclear DNA analyses have also shown a pattern of low nucleotide diversity (Karl et al., 1992).

With regard to hawksbill turtles, *Eretmochelys imbricata*, Broderick et al. (1994) first reported significant differences in mtDNA haplotype frequency between nesting areas in northeastern and northwestern Australia. In the Atlantic region, Bass et al. (1996) documented significant mtDNA haplotype frequency shifts among seven hawksbill rookeries. Espinosa et al. (1996) analyzed nesting samples from Cuba and Mexico by RFLP methods based on total mtDNA and a fragment of the mtDNA control, and showed that Mexican samples contained one haplotype found in no other Cuban samples. Koike et al. (1998) examined a longer sequence in the control region, and were able to discern important polymorphic sites, which allowed some haplotypes described by Bowen et al. (1996) and Bass et al. (1996) to be subdivided into new haplotypes.

In this paper, we present preliminary data on haplotypes detected from nesting and foraging populations of hawksbills from Caribbean Mexico, Cuba, and Puerto Rico. We establish genetic markers for rookeries and contribution rates for foraging samples from these areas. Haplotypes are based on sequence data of a 480 bp fragment of the mitochondrial control region.

MATERIALS AND METHODS

A total of 288 samples, including 70 nesting samples from Doce Leguas Cays and 218 foraging samples from 6 locations on the Cuban shelf (Fig. 1), were collected by the Cuban Ministry of Fisheries (MIP). Details of all DNA samples are listed in Díaz-Fernández et al. (1998).

Of the nesting samples, 58 were collected during nesting surveys carried out at Doce Leguas in 1994 and 1997 (24 and 34 samples, respectively; Fig. 2), and 12 were collected from shell plates from captive turtles raised at Isla de Pinos in 1995. These were assumed to have been transported as hatchlings from nests collected at Doce Leguas. The clutch of origin for these latter samples was not known, and it is likely that a number of animals were derived from the same clutches (Moncada et al., 1997a). Foraging samples were collected from 6 areas of Cuba (Fig. 1). From the southeast, 44 samples were collected during tagging surveys by research teams (23 and 13 from Doce Leguas in 1992 and 1997 respectively; 8 from Santa Cruz in 1993). A total of 115 foraging samples were collected from Isla de Pinos in the southwest during the traditional harvest (40 in spring of 1996

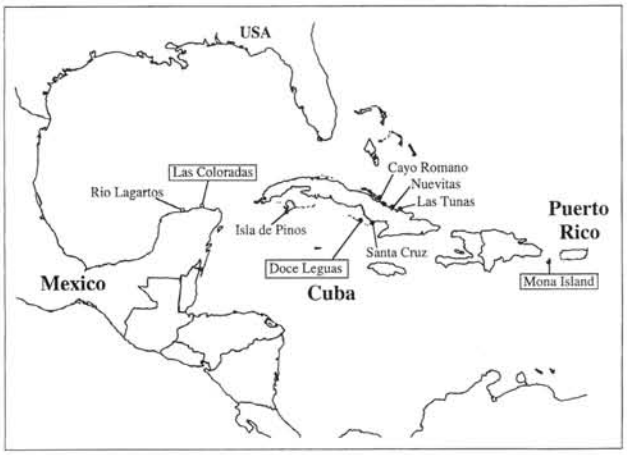

Figure 1. Locations of rookeries and foraging areas of hawksbills sampled in Cuba, Mexico, and Puerto Rico. Localities in boxes represent nesting and foraging populations, localities without boxes are only foraging populations.

and 1997; 75 in autumn of 1996). In the northeast, a total of 59 foraging samples were collected either during tagging studies being carried out there (17 from Nuevitas in 1992–93; 15 from Las Tunas in 1993–94) or from the traditional harvest (9 from Cayo Romano and 18 samples from the Northern Sea in 1995).

A total of 74 individual samples, gathered from 53 nesting animals and 21 foraging individuals were offered by the Mexican National Institute of Fisheries (Instituto Nacional de Pesca) for the project. In 1995 and 1996, 34 and 19 nesting samples, respectively, were collected at Las Coloradas, Yucatán, Mexico. These consisted of muscle, heart, or liver tissue from sacrificed neonates, which were preserved in 70% ethanol. Twenty-one foraging samples were collected at Río Lagartos, Yucatán. These were blood samples stored in an EDTA solution and preserved at 4°C.

Samples were supplied by the Turtle Research Program in Puerto Rico. A total of 126 samples from 20 hatchlings and 106 foraging individuals were collected from Mona Island. The foraging samples were blood.

When samples were taken from the inner surface of dorsal scutes, care was taken to avoid scraping the white wax-like residues on the surface. Soft tissue samples such as muscle, heart, liver, and skin were immediately preserved in 70% ethanol and stored at room temperature. Blood (about 1 ml) was taken from the cervical sinus of adult individuals and stored in a concentration of 50 mM EDTA solution at 4°C.

Either 10 mg of scute, approximately 20 mg of soft tissue, or 100 ml of blood was placed in 310 µl of RSB buffer, 15 µl of 10% SDS, and 25 µl of 20 mg/ml Proteinase k, and incubated for 2 hrs at 55°C on a rotator for protein digestion. Nucleic acids were extracted using an IsoQuick Nucleic Acid Extraction Kit (ORCA Research Inc., USA). Extracted DNA was amplified by the Polymerase Chain Reaction (PCR) method. Universal primer L15926 (5-TCAAAGCTTACACCAGTCTTGTAAACC-3) (Kocher et al., 1989) and sea turtle specific primer TCR6 (5-GTACGTACAAGTAAAAC-TACCGTATGCC-3) (Norman et al., 1994) were used to amplify the mitochondrial control region of the hawksbill. CONT1 (5-TGTACTATTGTACATCTACTTA-3), CONT2 (5-GTCACAGTAATGGGTTATTTCT-3), and CONT3 (5-TTTCTCGTGATGAGCTGAAC-3) were designed to amplify shorter fragments from the scute samples (Koike et al., 1998). The PCRs were performed with an ASTEC/Thermolyne

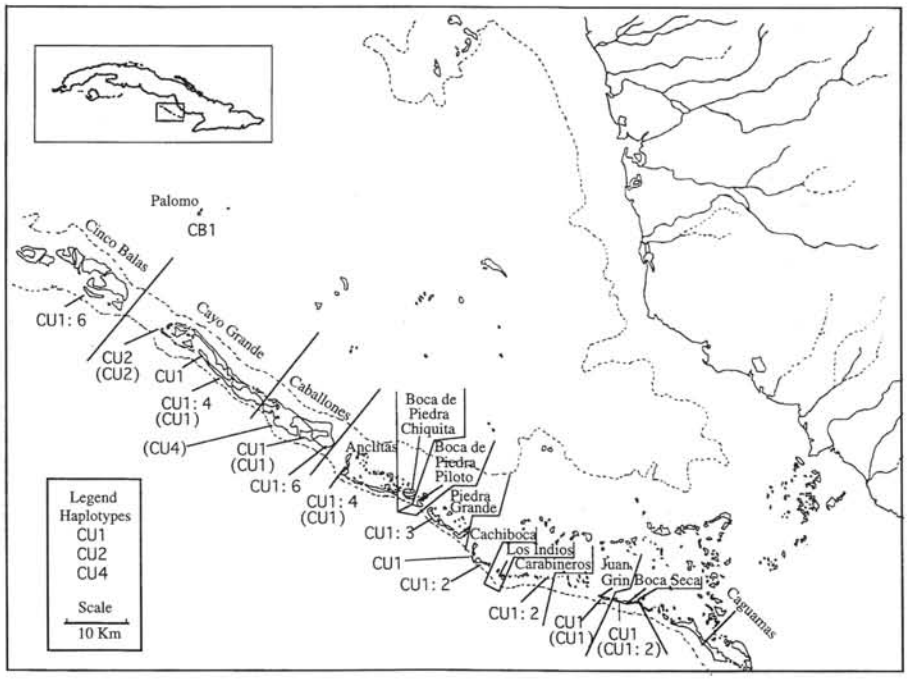

Figure 2. Map of the hawksbill nesting beaches in Doce Leguas Cays, Cuba, showing haplotypes of the nesting individuals in 1997 (no parentheses) and 1994 (in parentheses).

with 30 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 45 sec, and extension at 72°C for 45 sec.

Direct sequencing was undertaken in a DNA Processor (Pharmacia L.K.B. Co. Ltd.) with a Thermosequenase cycle sequencing kit (Amersham), using Cy5 fluorescent labeled primers of the same sequences used in the PCR. The cycle was repeated 20 times with denaturation at 94°C for 30 sec, annealing and extension at 65°C for 30 sec. Sequencing was performed with an ALFred DNA Autosequencer (Pharmacia L.K.B. Co. Ltd.). Alignment of the sequence data was accomplished with a BioResearch/AE program (Fujitsu Ltd.) based on CLUSTAL V (Higgins et al., 1992) and CLUSTAL W (Thompson et al., 1994) with gap penalty 2.

RESULTS AND DISCUSSION

From the 488 Caribbean samples (nesting and foraging), 28 polymorphic sites defining 30 haplotypes were observed in 480 bp fragments in the left domain of the mitochondrial control region (Table 1). These sequences were longer than those used by Bass et al. (1996), resulting in an additional 3 polymorphic sites at the 11th, 100th, and 124th bp from the beginning of the control region. Consequently, two haplotypes reported by Bass et al. (1996) were subdivided: F was divided into PR1, c, and j, and Q was divided into MX1 and MX2.

Haplotype frequencies for the three rookeries (Table 2) indicated that haplotype CU1 is the dominant haplotype in the nesting samples collected from Doce Leguas, Cuba, occurring in 88% of the samples. Less frequent were CU2, CU3, and CU4 (7%, 1%, and 1%, respectively). There was no significant difference in the haplotype frequencies between nesting samples collected in 1994 and 1997. Although only a few nesting locations were represented by the samples collected in 1994, it is interesting to note that nesting samples with CU2 were found at the same beach at Cayo Grande in both 1994 and 1997. Sampling locations from Doce Leguas in 1997 are shown in Fig. 2.

Nesting samples from Las Coloradas, Mexico, exhibited mainly haplotype MX1, representing 90% of the samples (Table 2). Haplotypes MX1a and MX2 were detected in samples collected in 1995, and haplotype MX3 in 1996.

Haplotype PR1 was the dominant haplotype in the Puerto Rican nesting samples collected from Mona Island in 1994 (60% of samples) (Table 2). The second most common haplotype was PR2 (30%), with haplotypes PR3 and PR4 making up the remainder.

The haplotype frequencies for these three Caribbean rookeries suggests that each nesting population has specific haplotypes as genetic markers. The one exception was PR1, the main haplotype for the Puerto Rican nesting population, which was also detected in one individual from the Cuban nesting population. This individual was sampled in the captive raising center at Isla de Pinos in 1995, and was assumed to have been transported from the rookery at Doce Leguas.

Bass et al. (1996), describing haplotype compositions from 7 rookeries in the Caribbean and western Atlantic regions, reported that haplotypes A and F were shared among nesting areas, and all other 19 haplotypes were unique to specific rookeries. Haplotype correspon-

Table 1. Haplotype table for 28 haplotypes recorded in the northern Caribbean region in this study, with comparison to the haplotype results of Espinosa et al. (1996), Bass et al. (1996), and Bowen et al. (1996). Haplotype MX1a, which has a 10 bp repeat (GCCTCTGGTT) at the 476th position, is not included in this table; haplotype Pac is not included in this table; haplotypes R, S, T, and U reported by Bass et al. (1996) are not included in this table; italic letters indicate transversions; = indicates indels; ** indicates no information on sequence data; parentheses around the number in polymorphic sites indicates no original report of substitutions.

No. from top of D-loop:			11	100	124	135	138	139	144	145	146	172	174	182	185	201	202	219	228	242	281	288	333	342	351	367	375	378	380	432	433	434	438	440	464
		Bass's:	(-114)	(-25)	(-1)	10	13	14	19	20	(21)	(47)	149	59	62	78	79	96	105	(119)	158	(164)	210	(219)	(228)	(244)	(252)	(255)	257	309	310	(311)	315	(317)	341
Espinosa	Bass	Koike	Polymorphic sites																																
	A	CU1	C	C	A	A	A	G	A	C	T	A	A	T	T	A	C	A	G	A	C	A	T	G	A	G	A	T	A	T	G	A	G	T	G
	G	i	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	O	PR4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
	α	g	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-
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	F	c	-	T	-	-	G	-	-	-	-	-	-	-	C	-	-	G	-	-	T	-	C	-	-	-	-	-	-	C	-	-	-	-	A
		j	-	-	G	-	G	-	-	-	-	-	-	-	C	-	-	G	-	-	T	-	C	-	-	-	-	-	-	C	-	-	-	-	A
		PR1	-	T	G	-	G	-	-	-	-	-	-	-	C	-	-	G	-	-	T	-	C	-	-	-	-	-	-	C	-	-	-	-	A
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3	Q	MX1	-	T	-	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	T	-	C	-	-	-	-	-	-	C	-	-	-	-	A
		MX2	-	T	G	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	T	-	C	-	-	-	-	-	-	C	-	-	-	-	A
	γ	CU2	-	T	G	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	T	-	C	-	-	-	G	-	-	C	-	-	-	-	A
		CU3	-	T	G	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	C	-	-	-	-	-	-	C	-	-	-	-	A
		CU4	-	T	G	-	G	-	-	-	T	-	-	-	C	G	-	G	-	-	T	-	C	-	-	-	-	-	-	C	-	-	-	-	A
		d	-	-	G	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	T	-	C	-	G	-	-	-	-	C	-	-	-	-	A
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		O	-	T	G	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	T	-	C	-	-	-	-	-	G	C	-	-	-	-	A
4	L	PR3	-	T	G	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	T	-	C	-	-	-	-	-	-	C	A	-	-	-	A
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		l	-	T	-	-	G	-	-	-	-	-	-	-	C	-	-	G	-	-	T	-	C	-	-	-	-	-	-	C	-	*T*	-	-	A
		p	-	T	-	-	G	-	-	-	-	-	-	-	C	-	-	G	-	-	T	-	C	-	-	-	-	-	-	-	-	-	-	-	-
		q	-	T	-	-	G	-	-	T	-	-	-	-	C	-	-	-	-	-	T	-	C	-	-	-	-	-	-	C	-	-	-	-	A

Table 2. Haplotype frequencies for three nesting populations in the northern Caribbean region.

Nesting Population	Samples	Haplotypes											
	n	CU1	CU2	CU3	CU4	MX1	MX1a	MX2	MX3	PR1	PR2	PR3	PR4
Doce Leguas, Cuba	70	62	5	1	1					1			
Breeding Center (1995)	12	9	2							1			
Doce Leguas (1994)	24	20	2	1	1								
Doce Leguas (1997)	34	33	1										
Mexico	53					48	1	3	1				
Las Coloradas (1995)	33					30	1	3					
Las Coloradas (1996)	19					18			1				
Mona Island, Puerto Rico	20									12	6	1	1

dence between our results and those of Bass et al. (1996) showed that haplotype F (detected from U.S. Virgin Islands, Belize, and Puerto Rico) could be subdivided into haplotypes PR1, c, and j, and we detected only haplotype PR1 from the Puerto Rican nesting population. Haplotype Q, detected by Bass et al. (1996) only from Mexico, was subdivided into MX1 and MX2, and both were found only in the Mexican nesting population. Haplotypes L, N, and O, detected only from Puerto Rico (Bass et al., 1996), corresponded to PR2, PR3, and PR4, while B and G from Antigua and Belize, respectively, corresponded to e and i, which we did not detect from the three rookeries analyzed. Haplotypes α and γ (Bowen et al., 1996), not detected from the seven rookeries examined by Bass et al. (1996), corresponded to g and CU2 respectively. All these data indicated that specific genetic markers were revealed for each rookery when more precise haplotypes were established by longer sequencing.

Using Maximum Likelihood Analysis, Bowen et al. (1996) estimated the contribution rate by the Mona Island, Puerto Rico, nesting population to the local foraging population as 12.7%. The low contribution rate reported by these authors seems mainly due to the low frequency of the main nesting haplotype in 1993 (F; 6.7%), compared with data from 1994 where the main nesting haplotype was detected in a high proportion of samples (PR1; 60%) (Koike et al., 1998). This suggests that different females appears in the nesting population each year, a concept supported by the ecological data that hawksbill nesting seasons are spaced at intervals of two or three years (Miller, 1997).

Haplotype frequencies for turtles from foraging grounds in the Caribbean region (Table 3) were examined using 218 samples from Cuba, 21 samples from Mexico, and 106 samples from Puerto Rico. Because haplotypes within the three nesting populations examined did not overlap (except for one individual in Cuba), contribution rates (Table 4) were calculated as a ratio of the individuals having the domestic nesting haplotypes to the total number of individuals analyzed.

In Cuba, foraging samples from the southeast consisted of three groups (from Doce Leguas collected in 1992 and in 1997 and from Santa Cruz in 1993). The contribution rates of Cuban nesting haplotypes in the foraging samples from southeastern Cuba were 83%, 54%, and 63%, respectively, with an average rate of 70%. Mexican and Puerto Rican nesting haplotypes in the foraging samples from southeastern Cuba were represented at 7% and 12%, respectively. The contribution of unknown haplotypes, not identified as one of the 3 sampled nesting populations, was 11%.

Foraging samples from Isla de Pinos in southwestern Cuba had a local nesting contribution rate of 46%; foraging samples from northeastern Cuba had local nesting contribution rates of 42%. It should be noted that one individual from the foraging sample in southwestern Cuba was identified to a haplotype in the cluster from the Pacific region (haplotype Pac) (Okayama et al., 1999).

There was also a high contribution rate of Mexican nesting haplotypes to the Mexican foraging samples (71%). There were no recorded Cuban nesting haplotypes detected in any of the foraging samples from Mexico. In Puerto Rico, Mona Island nesting haplotypes were moderately repre-

Table 3. Haplotype frequencies of foraging samples in Cuba, Mexico, and Puerto Rico. * Haplotype e corresponds to haplotype B by Bass et al. (1996) from rookery in Antigua. ** Haplotype i corresponds to haplotype G by Bass et al. (1996) from rookery in Belize.

Sampling Point	Samples	Haplotypes																								
	n	CU1	CU2	CU3	MX1	MX2	MX3	PR1	PR2	PR3	a	b	c	d	e*	f	g	h	i**	j	l	m	n	o	p	q	zz	Pac
Cuba																												
Southeast (Zone A)	44	28	1	2		3		5					1				2							1	1			
Doce Leguas (94TS105-127)	23	18	1			1		1																1	1			
Doce Leguas (96TS221-233)	13	5		2		2		1					1				2											
Santa Cruz (94TS154-162)	8	5						3																				
Southwest (Zone B)	115	46	5	2	4	7		24	10	1	2		10				1				1			1				1
Isla de Pinos: Spring (96TS234-268,348-358)	40	19	3	1	4	2		12	3	1			4											1				
Isla de Pinos: Autumn (96TS269-347)	75	37	2	1		5		12	7		2		6				1				1							
Northeast (Zone D)	59	18	5	2	5	3		13	3	2	1				1		2		1			1	2					
Nuevitas (94TS128-133, etc)	17	7			1	1		4		1	1						1					1						
Las Tunas (94TS134-139,142-150)	15	4	1			1		4		1					1		1		1				1					
Cayo Romano (95TS225-234)	9	2	1		3			2	1																			
Others (95TS205-224)	18	5	3	2	1	1		3	2														1					
TOTAL	218	92	11	6	9	13		42	13	3							5		1									1
Mexico																												
Rio Lagartos (1996)	21				12	1	2	1					1		1											3		
Puerto Rico																												
Mona Island (1994)	106	31			6	5		36	5	2		4	7	1	1	1	3	1	1	1							1	

Table 4. Haplotype contribution rates of nesting populations to foraging samples in the northern Caribbean region.

Foraging Samples	Samples	Haplotype Frequencies (%) by Nesting Population			
	n	Cuban	Mexican	Puerto Rican	Unknown
Cuba (Southeast)	44	70	7	12	11
Doce Leguas (94TS105-127)	23	83	4	4	9
Doce Leguas (96TS221-233)	13	54	15	8	23
Santa Cruz (94TS154-162)	8	63	0	38	0
Cuba (Southwest)	115	46	10	30	14
Isla de Pinos: Spring (96TS234-268, 348-358)	40	33	15	40	12
Isla de Pinos: Autumn (96TS269-347)	75	53	7	25	15
Cuba (Northeast)	59	42	14	31	13
Nuevitas (94TS128-133, etc)	17	41	12	29	18
Las Tunas (94TS134-139, 142-150)	15	33	7	33	27
Cayo Romano (95TS225-234)	9	34	33	33	0
Others (95TS205-224)	18	56	11	28	5
Mexico (Rio Lagartos)	21	0	71	5	24
Puerto Rico (Mona Island)	106	29	10	41	20

sented in the local foraging samples (41%), with Cuban nesting haplotypes also being well represented (29%), in addition to 10% contribution from Mexico. Although Bowen et al. (1996) estimated the local contribution rate of the foraging population at Mona Island as 12.7%, the low estimate of the contribution rate seems mainly due to the low frequency of the main Puerto Rican nesting haplotype. When haplotype F was counted as the main Puerto Rican nesting haplotype, there were no significant differences ($p > 0.05$) between foraging samples collected in 1993 (53%) and those in 1994 (41%).

This DNA study is an integral part of an overall program, which includes other key components such as reproduction (Moncada et al., 1997a), tagging, and movements (Moncada et al., 1997b), and further investigations should be continuing. Haplotypes of unknown sources, found to reach about 15% in the foraging samples, need further analysis of additional nesting populations.

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