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Genetic diversity of *Chelonibia caretta*, commensal barnacles of the endangered hawksbill sea turtle *Eretmochelys imbricata* from the Caribbean (Puerto Rico)

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The patterns of mitochondrial genetic diversity were studied in Chelonibia caretta*, commensal barnacles of the endangered hawksbill sea turtle* Eretmochelys imbricata *from Mona Island of Puerto Rico. Two mitochondrial genes were sequenced: the large subunit of ribosomal RNA (16S rRNA) and the cytochrome oxidase subunit I (COI). Haplotypic diversity (h) for 16S ($N = 34$) and COI ($N = 26$) varied from 0.763 to 0.468, respectively. The nucleotide diversity (π) of 16S/COI (0.00284/0.0013) of* C. caretta *was low compared to all other published sequences from population studies of barnacles attached to fixed substrate. However, the estimated levels of π from* C. caretta *were comparable to those reported from* C. testudinaria*, an epibiont barnacle on loggerhead turtles. Analysis of molecular variance and parsimony network analysis of the sampled turtle epibionts from Mona Island indicated that they are genetically homogeneous, irrespective of the host or its gender. Albeit that the sampling size is small, our results are consistent with the general prediction that higher rates of dispersal reduce population divergence and estimates of nucleotide diversity. Conservation concerns are raised about the status of* Chelonibia caretta *whose host (the hawksbill sea turtle) has been included in the Endangered Species List.*

Keywords: turtle barnacles, hawksbill turtle, mtDNA, Puerto Rico, Caribbean

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INTRODUCTION

A conspicuous component of the marine turtle epibiotic community is the coronulid barnacle of the genus *Chelonibia*. Twenty-nine nominal species of 'turtle barnacles' are currently recognized (Epibiont Research Cooperative, 2007). These represent two balanomorph families: the Chelonibiidae (acorn barnacles) and the Platylepadidae (gooseneck barnacles). Studies of hawksbill sea turtle (*Eretmochelys imbricata*) epibiota revealed that two common obligate commensal barnacle species attached to the carapace are *Chelonibia caretta* and *C. testudinaria* with the former being the most common (Frick *et al.*, 2003; Schärer, 2003).

Although the life history of *C. caretta* remains unknown in terms of dispersal potential, the complete life cycle of the congeneric *C. testudinaria* has been described by Zardus & Hadfield (2004), who documented that development from the first naupliar stage to the cyprid stage occurs in nine days. In laboratory assays, barnacle settlement occurred one or two days after completing development, however, it remains unknown how long *C. testudinaria* can remain in the cyprid stage in nature. Other barnacle species settle within two to six weeks (Lucus *et al.*, 1979; Shanks, 1986; Pineda *et al.*, 2005).

One of the largest nesting locations for hawksbills in the Caribbean Sea is Mona Island, Puerto Rico (Beggs *et al.*, 2007; van Dam & Diez, personal communication). Tagged adult hawksbill turtles from Mona Island, are capable of travelling to foraging habitats as geographically distant as Central America and the British Virgin Islands (van Dam *et al.*, 2008). Unlike barnacles on fixed substrates, turtle barnacles are attached on a mobile substrate and are capable of dispersing both as larvae and as adults. Higher rates of dispersal should prevent significant population subdivision as populations cease diverging due to drift or local adaptation. We have evaluated this hypothesis by analysing the genetic variation at the mitochondrial genes COI/16S (914 bp) from 34/27 *C. caretta* collected from Mona Island and have compared our nucleotide diversity levels to previous population studies of barnacles inhabiting fixed substrates.

MATERIALS AND METHODS

Barnacles were sampled from several adult and juvenile hawksbills at Mona Island, Puerto Rico a known breeding location of this species. The sampling sites were located on the southern coast of Mona Island (18°5′27″N and 67°53′37″W). Barnacles were collected from nesting and

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Table 1. Barnacle haplotypes and relative positions segregating sites from 914 bp of the concatenated sequences of the mitochondrial 16S and COI genes sampled across 35 specimens from 3 female (♀), 6 male (♂), and 1 juvenile (Juv) hawksbill turtles. Hap., haplotypes; -, gaps; ?, missing data; N, number of barnacles sharing this haplotype. The Specimen ID column refers to the GenBank codes of sequenced barnacles as well as the gender of their respective turtle host.

Haplotype	16S (389 bp)														COI (525 bp)						N	Specimen ID
	46	72	76	78	187	221	270	276	277	278	300	312	335	388	417	537	597	711	813	856		
1	C	C	A	A	-	A	A	T	A	A	A	A	T	G	G	G	T	C	G	A	1	♀1_1
2	.	T	.	.	-	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	1	♀1_2
3	.	.	.	.	-	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	4	♀1_3, ♀3_1, ♂2_1, Juv 1
4	.	.	G	.	-	.	.	.	.	.	.	-	.	.	.	.	.	.	.	G	1	♀1_4
5	.	.	.	.	-	.	.	.	T	.	.	.	.	.	A	.	.	.	.	G	2	♀1_5, ♂6_3
6	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	9	♀1_6, ♀1_7, ♀2_1, ♀2_2, ♂1_3, ♂1_5, ♂5_3, ♂3_1, ♂5_1
7	T	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	1	♀1_8
8	.	.	.	.	-	.	.	.	.	.	.	.	.	A	.	.	.	.	.	G	1	♀1_9
9	.	.	.	.	-	G	.	.	T	.	.	.	.	.	.	.	.	.	.	G	1	♀1_14
10		.	.	.	-	.	G	.	T	.	.	.	.	.	.	.	.	.	.	G	1	♀3_2
11	.	.	.	.	-	.	.	.	.	.	C	.	.	.	.	.	.	.	A	G 1	1	♀3_3
12	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	C	T	.	G 5	5	♂2_4, ♂6_3, ♀1_10, ♀1_12, ♂1_2
13	.	.	.	.	-	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	3	♂3_2, ♂4_1, ♂4_2
14	.	.	.	G	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	1	♂6_2
15	.	.	.	.	A	.	.	.	.	.	.	.	.	.	?	?	?	?	?	?	1	♀1_13
16	.	.	.	.	-	.	.	.	.	.	.	.	A	.	?	?	?	?	?	?	1	♂1_1
17	.	.	.	.	-	.	.	.	T	T	.	.	.	.	?	?	?	?	?	?	1	♂2_2
18	.	.	.	.	-	.	.	A	.	.	.	.	.	.	?	?	?	?	?	?	1	♂2_3

foraging turtles during August and September of 2006 and 2007. *Chelonibia* sp. barnacles attached to the carapace were removed from three females (17 barnacles) and six males (18 barnacles). One of these barnacles was collected from a juvenile turtle from Culebra Island, eastern Puerto Rico. A specimen of *Chelonibia testudinaria* was collected from another juvenile turtle from Mona Island and was used as outgroup in the genealogical analysis. Barnacles were preserved in 100% ethanol for DNA analysis.

Total genomic DNA was extracted using a Gentra DNA purification kit (Qiagen, Inc.). A fragment of the 16S rRNA gene was amplified using primers from Crandall & Fitzpatrick (1996). A fragment of the COI was amplified with the universal primers from Folmer *et al.* (1994). Amplification was performed using the Master Taq® kit. To amplify the COI and the 16S gene, the thermal cycler profile began with a denaturation step at 94°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, 45°C for 30 seconds (COI; 42°C for 50 seconds), and 72°C for 30 seconds (COI; 1 minute), ending with an extension step at 72°C for 5 minutes. Some DNA templates that were difficult to amplify were further cleaned by a Qiagen PCR Clean Up Kit and Mg^{+2} hot beads. PCR products were visualized by electrophoresis on a 1% agarose gel, and cleaned using ExoSap. Sequence reactions were prepared with the BigDye Terminator v.3.1 Cycle Sequencing Kit for both strands of the purified amplicon and loaded in an ABI 3130xl Genetic Analyzer. The resulting DNA sequences were verified by aligning reads from both 5′ and 3′ directions for the majority of individuals, using the software CodonCode Aligner and further curated in MacClade (Maddison & Maddison, 2000). The haplotypic (h) and genetic diversity (π) (Nei, 1987) of barnacles was estimated in DNAsp (Rozas *et al.*, 2003). The DNA neutrality tests Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) were applied to test for significant departures from neutrality (Kimura, 1968). Additionally, a hierarchical analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was applied to detect population differentiation between barnacles collected from male and female turtles. AMOVA and pairwise Φ_{ST} comparisons were performed in Arlequin ver. 3.1 (Excoffier *et al.*, 2005) by using the K2P + Γ (0.222) (Kimura, 1980) model of nucleotide substitution. We used the K2P model to increase resolution in our data set which was characterized by many closely related haplotypes. The significance of Φ_{ST} was assessed by 10,000 permutations of groups and haplotypes. A parsimony haplotype network was constructed for the concatenated sequences using the Templeton *et al.* (1992) algorithm as implemented in TCS v.1.21 (Clement *et al.*, 2000). Genealogical trees of the mitochondrial sequences were built with the maximum parsimony method in MEGA 4 (Tamura *et al.*, 2007). DNA sequences have been deposited in GenBank under Accession Numbers FJ385704–FJ385766.

RESULTS

After verifying and trimming the DNA traces, a 389 bp segment of the mitochondrial gene 16S from 34 individual barnacles and a 525 bp segment of COI from 27 barnacles were included in the analysis. Two barnacles from turtles Female 1 and Male 1 did not yield high quality sequences for either gene. We identified 18 different haplotypes (Table 1). Four haplotypes of barnacles (Hap 3, 5, 6 and 12) were shared by female and male turtles, while nine haplotypes were found only on females and six only on males (Table 1). Of the 18 haplotypes, 13 were singletons. There were 14 segregating sites (including two indels) in 16S and six segregating sites in COI. There were seven transitions and five transversions in 16S while all nucleotide substitutions in COI were transitions. Haplotypic diversity (h) of barnacles ranged from 0.822 to 0.933 for male and female turtle hosts, respectively for the concatenated data (Table 2). Nucleotide diversity (π) ranged from 0.00198 to 0.00213 for males and females, respectively for the concatenated data (Table 2). There were no significant differences in estimates of h and л when barnacles were grouped according to the gender of the host, for neither gene nor the combined data (Table 2).

When we included all barnacles with complete sequences (16S + COI, N = 25), the increased number of singletons resulted in significant deviations from neutrality (Tajima's $D = -1.92072$, $P < 0.05$; Fu's $Fs = -10.675$). The significantly negative values of Tajimas's D and Fu's Fs tests are consistent with an expanding population or a purifying selection scenario. When we applied the tests gene-by-gene, we detected significant deviations from neutrality with 16S but borderline non-significant with COI, as most of the singletons were observed in the 16S region.

When barnacle sequences were partitioned by individual hosts, AMOVA indicated that most of the genetic variation of barnacles (98.24%) was observed within individual turtles, suggesting that the sampled *C. caretta* were not significantly differentiated between hosts ($\Phi_{ST} = 0.01761$, $P = 0.408 \pm 0.018$). When partitioned by gender of the host turtles, AMOVA suggested that most of the genetic variance (89.52%) was observed within rather than among gender for

Table 2. Host gender estimates of *Chelonibia caretta* haplotypic diversity h (±SD) and nucleotide diversity π (±SD) for 16S and COI, separately and combined (16S + COI).

Gene	Host sex	No. Host (turtles)	No. (barnacles)	h	π
16S	Female	3	18	0.797 (0.090)	0.00311 (0.00059)
	Male	6	16	0.742 (0.084)	0.00258 (0.00051)
	Total	9	34	0.763 (0.063)	0.00284 (0.00041)
COI	Female	3	15	0.476 (0.155)	0.00102 (0.00038)
	Male	5	11	0.473 (0.473)	0.0016 (0.00062)
	Total	8	26	0.468 (0.118)	0.0013 (0.0004)
16S + COI	Female	3	15	0.933 (0.054)	0.00213 (0.00034)
	Male	5	10	0.822 (0.097)	0.00174 (0.00038)
	Total	8	25	0.883 (0.052)	0.00198 (0.00029)

the concatenated genes. The overall $\Phi_{ST} = 0.1048$, which is not significant ($P = 0.077 \pm 0.003$). The population specific Φ_{ST}s were 0.1148 for barnacles attached to males and 0.0981 for barnacles attached to females. There was no genetic differentiation driven by the gender of the host, regardless if we concatenated or separated the genes. Pairwise Φ_{ST} comparisons did not indicate significant genetic differentiation of barnacles among hosts or gender of hosts (data not shown). The haplotype network analysis reflected the plethora of singletons and the lack of genetic population structure in the barnacles (Figure 1). The haplotype relationships as depicted in the network analysis are consistent with a demographic scenario of population expansion or purifying selection. Genealogical analysis of the concatenated 16S + COI sequences (914 bp) resulted in a mostly unresolved topology (data not shown).

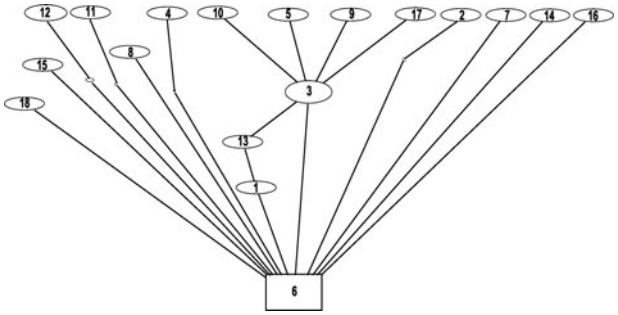

Fig. 1. Parsimony haplotype network based on the concatenated mitochondrial sequences COI + 16S of *Chelonibia caretta*. The size of ovals is proportional to the observed number of sequences for that corresponding haplotype (see Table 1 for haplotypic frequency and definition). The minimum number of steps is represented by the small empty circles between each haplotype. The boxed haplotype (6) is the hypothesized root of the network and the closed loop represents homoplasy, as indicated by the TCS analysis (Clement *et al.*, 2000).

DISCUSSION

Genetic diversity is the most basic unit of ecosystem diversity, playing a central role in how populations/species respond to changing environments. Comparisons of genetic diversity levels between *Chelonibia caretta* (Table 2) with those reported from barnacle species settled on fixed substrates (e.g. rocks, piers and mangrove roots) reveals that the commensal barnacles harbour low levels of genetic variability (Table 3). *Chelonibia caretta* exhibited similar to slightly lower values of genetic diversity than the congeneric *C. testudinaria* (0.002–0.008; Table 3), but the latter commensal species was collected from a much wider geographical region and from a different turtle host species (Rawson *et al.*, 2003). The mitochondrial gene COI is the most frequently sequenced gene in population studies of barnacles and comparisons of nucleotide diversities reveal that previously reported values are about an order of magnitude higher than those of *C. caretta* (Table 3). Nucleotide diversities from other mitochondrial and nuclear genes yield similar or higher genetic diversities. However, comparisons may not be appropriate

Table 3. Estimates of genetic diversity (π) of barnacle species from the literature. When population information was available, the range of genetic diversity is provided (CR[#]). Studies containing less than 5 sequences per species were not included. Studies that did not include GenBank submission number were not included. Sequences also include flanking rRNA and tRNA sequences.

Species	π	Gene	Source	Accession Numbers
Balanus glandula	0.0113 to 0.0188	COI	Sotka *et al.* (2004)	AY62954–AY630026
	0.0072 to 0.0106	COI	Wares *et al.* (2001)	AF234351–AF234462
Chelonibia testudinaria	0.0020 to 0.0080	COI	Rawson *et al.* (2003)	AY174289–AY174367
Chthamalus proteus	0.0110 to 0.0230	COI	Zardus & Hadfield (2005)	AY822764–AY823025
Chthamalus fissus	0.0112 to 0.0144	COI	Wares *et al.* (2001)	AF234463–AF234527
Chthamalus dalli	0.00072*	COI	Wares & Castañeda (2005)	AY795282–AY795480
Chthamalus stellatus	0.01226*	Ef1-a	Shemesh/Achituv[&]	AM396207–AM396225
Chthamalus montagui	0.00265*	Ef1-a	Shemesh/Achituv[&]	AM396226–AM396249
Pollicipes pollicipes	0.0319 to 0.0669	CR[#]	Quinteiro *et al.* (2007)	AY939545–AY939779, EF029129–EoF29178.
	0.07297	COI	Quinteiro *et al.* (2007)	EF032142–EF032154
Semibalanus balanoides	0.0520 to 0.0870	CO1	Brown *et al.* (2001)	AF385899–AF385925
	0.00993[†] to 0.01569[††]	COI	Wares & Cunningham (2001)	AF242660–AF242728
Tetraclita pacifica	0.0000	ITS-1	Chan *et al.* (2007a)	DQ363725–DQ363740
	0.0431*	12S	Chan *et al.* (2007a)	DQ363711–DQ363717
	0.00860 to 0.01190	COI	Chan *et al.* (2007a)	DQ363680–DQ363695
Tetraclita squamosa	0.0000*	ITS-1	Chan *et al.* (2007a)	DQ363741–DQ363746
	0.00324*	12S	Chan *et al.* (2007a)	DQ363718–DQ363721
	0.00667	COI	Chan *et al.* (2007a)	DQ363696–DQ363706
Tetraclita singaporensis	0.00771	COI	Chan *et al.* (2007b)	EF035162–EF035167
Tetraclita japonica	0.01002 to 0.01289	COI	Tsang *et al.* (2007)	DQ647704–DQ647742
	0.03749 to 0.06475	CR	Tsang *et al.* (2007)	DQ645847–DQ645887
Tetraclita formosana	0.00943	COI	Tsang *et al.* (2007)	DQ647743–DQ647768
	0.04789	CR	Tsang *et al.* (2007)	DQ645827–DQ645846

(†) π value was generated by pooling all European populations together. (††) π value was generated by pooling all North American populations together. (*) π value was generated by pooling all sequences of the study because sampled populations consisted of fewer than 5 individuals. ([&]) Unpublished manuscript. CR, control region; Ef1-a, elongation factor 1 alpha.

since different genes are usually under different selection constraints.

The population dynamics of hosts and their epibionts should be tightly linked. Barnacles attached to fixed substrates are capable of dispersing during the larval stage whereas barnacles attached to mobile substrates (e.g. turtles, boats and driftwood) can disperse during both larval and adult stages. Because *C. caretta* is an obligate commensal of marine turtles, its dispersal potential should be influenced by the migratory behaviour of its hosts, as it has been shown for the congener *C. testudinaria* (Rawson *et al.*, 2003). The parsimony network analysis and the molecular variance of the sampled barnacles indicate that they are genetically homogeneous, irrespective of the host or its gender. Such pattern of molecular variance suggests that barnacles settle on turtles in mating and/or foraging areas, where both sexes can be found concomitantly. Alternatively, rates of gene flow between barnacle populations are high enough to result in panmixia. The nesting population of Mona Island is genetically distinct from other Caribbean rookeries (Díaz-Fernandez *et al.*, 1999; Velez-Zuazo *et al.*, 2008), but the hawksbills feeding in the adjacent coral reef habitats are genetically composed of turtles from geographically distinct rookeries (Bowen *et al.*, 1996). Because our samples were collected from Mona Island (and only one from Culebra Island) absence of population structure may not be indicative of the demography of the Caribbean populations of the barnacle. Additional sampling of *C. caretta* from genetically distinct Caribbean rookeries will be sufficient to test this hypothesis. On the other hand, the complex life history of the hawksbill turtle with ontogenetically onset migration, repeated movements between foraging, breeding and nesting habitats and large variation of migratory behaviour between and within sexes (van Dam *et al.*, 2008; Velez-Zuazo *et al.*, 2008) may explain the absence of population structure in the commensal barnacles. Significant recruitment to foraging habitats of Mona Island from other Caribbean rookeries (Velez-Zuazo *et al.*, 2008), exposes resident hawksbills to migrant hawksbills with epibiont loads from other Caribbean regions. If the exposure to allochthonous turtles through habitat sharing is long enough, then a mechanism is provided to genetically homogenize the epibiont barnacle populations. In foraging habitats, cyprids from different geographical origins can settle equally on resident and non-resident feeding turtles regardless of the host or its gender. The success of cyprid settlement on the host carapace depends on the density of the hosts and barnacles, and the period of exposure of the host to the barnacles (Zardus & Hadfield, 2004). Based on our results, the most likely time and place for cyprid settlement should be nearshore habitats where turtles aggregate to mate or forage.

The hawksbill sea turtle *Eretmochelys imbricata* has declined as much as 80% during the last century (Meylan & Donelly, 1999) and has been listed as critically endangered by the International Union for the Conservation of Nature (Baillie & Groombridge, 1996). Any reductions of the host population will be accompanied by a reduction of the epibiont populations, since hawksbill turtles constitute the primary habitat. The hawksbill turtle populations in the Atlantic are depleted (Meylan, 1999), and with an estimated female census size of 5000 turtles in the Caribbean (McClenachan *et al.*, 2006), the number of suitable turtle hosts/habitats for barnacles is now limited. Because turtle barnacles have unique life histories tightly linked to their host's life history, they are susceptible to the same demographic processes, such as rapid population declines. Even though the observed excess of singletons in the mtDNA of barnacles may be explained by a population expansion, this pattern represents a genetic footprint of the past, when host populations were abundant. Similarly, other marine species (e.g. the sea urchin *Diadema antillarum* (Lessios *et al.*, 2001) and whales (Roman & Palumbi, 2003)) that have undergone recent drastic declines, harbour patterns of genetic variation indicative of much higher past population sizes than the current ones.

The US Endangered Species list comprises primarily host species; however, these species are interconnected or affiliated with many other organisms (predators, preys, parasites, commensals, etc.). As many as 6300 affiliated species have been estimated to be co-endangered along with the host species (Koh *et al.*, 2004), indicating how closely linked the evolutionary destinies of affiliate–host groups are. The *Chelonibia caretta–Eretmochelys imbricata* is an affiliate–host species pair where the host has been included in the Endangered Species List; therefore, we suggest that the barnacle *C. caretta* should also be considered a candidate for listing due to drastic declines of its primary habitat.

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