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Microbial community structure in sand on two olive ridley arribada nesting beaches, Playa La Flor, Nicaragua and Playa Nancite, Costa Rica

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ABSTRACT

Microbial relative abundance, diversity and richness were assessed based on 16S rRNA gene-based TRF community analysis via terminal restriction fragment (TRF) analysis on two olive ridley turtle arribada nesting beaches in Central America, Playa La Flor in Nicaragua and Playa Nancite in Costa Rica. Arribada beaches have very high densities of egg clutches in small areas, and the sand contains numerous broken eggs due to turtles inadvertently disturbing previously laid clutches. The high organic content (broken eggs) in the sand causes microbial build up in clutches, which in return may affect hatching success. Phylotype relative abundance (fragment abundance) differed with nest density and distance from the water at both Playa Nancite and Playa La Flor. Higher relative abundance of certain bacterial species in high nest density areas may have contributed to lower hatching success. Bacterial diversity and richness increased with nest density and were higher in the zones closer to vegetation suggesting that bacterial diversity and richness may be important factors affecting hatching success of olive ridley eggs on these nesting beaches. To better conserve olive ridley sea turtles and manage egg harvest on arribada nesting beaches further studies should focus on identifying bacterial species that are pathogenic to turtle eggs and their effects on hatching success.

1. Introduction

Nearly all sea turtles are threatened, endangered or critically endangered by combination of natural and anthropogenic factors notably including loss of nesting habitat and loss or take of deposited eggs (Cornelius, et al., 2007; Lutcavage et al., 1997; Witherington and Frazer, 2003). One of the critical processes in maintaining sea turtle population is increasing hatching success of incubating eggs (Hamann, et al., 2010). Sustained hatching success on a sea turtle nesting beach is influenced by many factors, including density dependent nest destruction (Caut, et al., 2006; Honarvar, et al., 2008; Tiwari, et al., 2006), egg predation (Fowler, 1979), egg take (Cornelius, et al., 2007), temperature (Ackerman, 1996), gas exchange (Ackerman, 1996; Honarvar et al., 2008; Maloney, et al., 1990; Wallace, et al., 2004), moisture (McGehee, 1990), and microbial load in the nest environment (Cornelius, 1986; Wyneken et al., 1988). Understanding how each factor affects hatching success is a fundamental part of any beach management project (Hamann, et al., 2010).

Microorganisms can be important selective forces in the evolution of oviparous animals through developmental interference and mortality (Mills et al., 1999; Nuttall, 1997; Pinowski et al., 1994). Fungi and/or gram-negative bacteria on bird (Kozlowski et al., 1991; Lombardo et al., 1996; Stewart and Birch Rambo, 2000) and reptile (Ewert, 1979) eggshells in some instances have been shown to decrease water resistance of the shell by digesting the cuticle and also facilitate microbial infection leading to reduced hatching success (Board et al., 1979; Cook et al., 2005).

Using culture-dependent methodologies, the presence of microorganisms on the egg exterior or in embryonic tissue has been described in several species of sea turtles including the loggerhead turtle (*Caretta caretta*) (Awong-Taylor et al., 2008; Peters et al., 1994; Ragotzkie, 1959; Wyneken et al., 1988), the green turtle (*Chelonia mydas*) (Bustard and Greenham, 1968; Solomon and Baird, 1980; Whitmore and Dutton, 1985), the leatherback turtle (*Dermochelys coriacea*) (Eckert and Eckert, 1990; Solomon and Tippett, 1987; Whitmore and Dutton, 1985) and the olive ridley turtle (*Lepidochelys olivacea*) (Acuña-Mesén, 1992; Acuña-Mesén et al., 1999; Mo et al., 1990; Mo et al., 1992). In addition, it has been speculated that infection is an important source of egg failure (Cornelius, 1986). Despite the potential importance of microbes to hatching success, microbial diversity and relative abundance have not been explored on natural sea turtle nesting beaches.

Olive ridley turtles nest in high-density events referred to as "arribadas" at only a few nesting beaches in the world (Bernardo and Plotkin, 2007; Cornelius et al., 1991). The most important nesting beaches are in Costa Rica (Playa Ostional and Playa Nancite), Mexico (La Escobilla), India (Orissa) and Nicaragua (Playa Chacocente and Playa La Flor). Numbers of olive ridley nests seem to be increasing at Playa La Flor (46,000 in 1999, 71,000 in 2004 to 167,000 in 2006) but decreasing at Playa Nancite (340,000 clutches in 1981, 200,000 in 1982, to 13,000 in 2005 and 18,000 in 2007) (Cornelius et al., 1991; Fonseca et al., 2009; Honarvar, 2007; Plotkin et al., 1997). One potential cause for the

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population decline at Playa Nancite is low egg survival resulting in decreased recruitment. Arribada nesting results in destruction of large numbers of clutches by turtles that disturb previously laid clutches (Bernardo and Plotkin, 2007; Cornelius, 1986). The large number of broken eggs increases the organic content and resources for microbes, which in return increases microbial abundance in the sand where the eggs are incubating (Cornelius, 1986; Cornelius et al., 1991). Microbes can alter the nest environment, compete for resources, and also directly infect incubating eggs. Cornelius (1986) speculated that egg removal may increase hatching success by reducing the number of decomposing eggs in the sand. This speculation is used in management programs for olive ridley arribada beaches around the world (Campbell, 1998; Campbell, 2007; Cornelius et al., 1991; Hope, 2002). Yet it is not known whether microbial diversity and relative abundance differ at different nest densities or on different parts of a nesting beach. If microorganisms affect hatching success then microbial studies are crucial to guide management decisions for these important arribada nesting beaches.

Since many microorganisms are not easily cultured, polymerase chain reaction (PCR)-amplified rRNA genetic techniques are necessary to study changes in microbial community structure and diversity (Avaniss-Aghajani et al., 1994; Head et al., 1998). Terminal restriction fragment length polymorphism (TRF, or T-RFLP) is a DNA-based analysis that allows rapid comparison of complex bacterial communities. TRF measures differences in the positions of restriction sites in specific DNA sequences from different microbes. The fragments allow us to estimate the number of microbial species found in a sample (Hackl et al., 2004). TRF has been used to study changes in microbial structure, diversity and relative abundance in agricultural soil, grassland forest soils, biological soil crusts and in coastal environments (Hackl et al., 2004; Nogales et al., 2007). A possible limitation of TRF analysis is that each peak in a profile could represent several TRFs of the same size, originating from different 16S rRNA genes thus underestimating microbial diversity. Regardless, TRF is still a useful method to assess the similarity of soil bacterial communities allowing spatial heterogeneities and temporal changes to be detected in highly diverse bacterial communities without the need to identify every peak in every profile (Lukow et al., 2000). In addition, underestimation can be minimized by using the appropriate number and types of restriction endonucleases, resulting in TRF profiles that more accurately reflect the natural diversity of microbial population (Engebretson and Moyer, 2003; Osborne et al., 2006).

In this study, 16S rRNA TRF community analysis was used to assess the relative abundance and diversity of bacteria on olive ridley nesting beaches. Two questions were addressed: 1) do microbial community diversity and relative abundance differ in sand on different parts of two arribada nesting beaches frequented by olive ridleys, Playa La Flor and Playa Nancite? 2) Do diversity and relative abundance of bacterial communities differ in areas with different nest densities on these two beaches?

2. Methods

2.1. Site description and sampling

Sand was collected at the end of olive ridley nesting season at Playa Nancite, Costa Rica in January 2007 and Playa La Flor, Nicaragua in February 2007. Playa La Flor is a 1.6 km long beach situated on the southern Pacific coast of Nicaragua. Playa Nancite is 1.1 km long beach located on the northwest Pacific coast of Costa Rica in Guanacaste Province. Both nesting beaches are 15 to 20 m wide, and unstable in that beach profiles change with storms.

Sand samples were taken from areas with different nest densities and from different beach zones (Table 1). The width of the beach was divided into three zones: the high beach zone was the area closest to the vegetation (0–5 m), the middle zone was 5–10 m from vegetation and the low zone on the beach was the area closest to the high tide line, 10–15 m from vegetation. Nest density samples were divided as follows: high estimated nest density (~20 nests/m^2), moderate nest density (~10 nests/m^2), low nest density (~5 nests/m^2) and a control (0 known nests/m^2). Nest densities were estimated using total number of clutches laid on the beach during November, December 2006 and January 2007 per sampled area at Playa Nancite and Playa La Flor as estimated by park rangers who counted the number of nesting turtles present on different parts of the beach. The turtles were counted individually and marked with a dab of white paint on their carapace. All clutches were potentially still viable and not yet hatched. Since it was not possible to distinguish clearly using the rangers estimates between low and moderate nest densities at Playa Nancite or between high and moderate densities at Playa La Flor, those categories were combined in the design (Table 1). Due to the large numbers of nests on the beach, we cannot guarantee that the control sites were completely devoid of nests. At Playa La Flor turtles used the entire beach and no nest free controls were possible. Three replicate sand samples were collected at nest depth (30–35 cm depth) randomly from each site by digging down to nest depth (using sterile gloves) by hand. Each sample within a particular zone was >50 m from other. For each sample, three 50 ml sub-samples of sand from each hole were collected. Sand samples were transported to the laboratory and stored at −20 °C within 24 h of collection.

Table 1
Sampling sites and nest densities from which sand samples were collected from Playa Nancite and Playa La Flor in 2007. Samples were taken at high, middle and low zone beach. Each sample consisted of three subsamples at each site. No samples were taken from middle zone of the beach in low nest density areas at Playa La Flor.

Site	Nest density		
	High	Moderate	Low
Playa Nancite	X	X	–
Playa La Flor	X	–	X

2.2. Microbial community DNA extraction

DNA was extracted from sand samples using a modified PowerMax™ soil DNA isolation kit (MoBio, Solana Beach, CA, USA). Two grams of each sub-sample from a hole was pooled and mixed in a 50 ml sterile tube and DNA was extracted. This step was repeated three times and each time the DNA was collected in a separate sterile tube. To ensure high yields of genomic DNA, power bead solution from the kit was added to the sand samples followed by three freeze (−20 °C)–thaw (60 °C) cycles. The DNA of each replicate was quantified using a spectrophotometer (Spectronic, Genesys 2.0). DNA from all replicates from the same nest cavity was mixed and the DNA was re-quantified.

2.3. PCR with 16S rRNA gene primers

The extracted DNA served as template for PCR using 16S rRNA primers: 8F primer labeled with 6-carboxyfluorescein (6-FAM 5′ AGAGTTTGATCCTGGCTCAG 3′) and 926R (5′ CCGTCAATTCCTTTRAGTTT3′) (Hackl et al., 2004; Muyzer et al., 1995). The PCR mixture (50 μl) contained 60 to 120 ng of extracted DNA, 1X reaction buffer, dNTP (200 μM), $MgCl_2$ (0.5 mM), primers (0.2 μM) and 0.5 units of *Taq* DNA polymerase (Boehringer). Three independent PCRs were performed for each sample as follows: initial denaturing step of 5 min at 95 °C, 30 cycles of denaturing, annealing and extension (30 s at 95 °C, 1 min at 53 °C and 2 min at 72 °C respectively) followed by a final extension step of 10 min at 72 °C. The three PCR products from the same sample were pooled to reduce PCR bias and the products were purified with a MinElute PCR purification kit (Qiagen Inc., Chatsworth, CA, USA) with final elution volume of 20 μl.