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High frequency of multiple paternity in a solitary population of olive ridley sea turtles in Honduras

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ABSTRACT

Females of all seven living species of sea turtles are known to be polyandrous and show multiple paternity. The frequency of multiple paternity varies among species, and among populations of the same species. In the olive ridley sea turtle (*Lepidochelys olivacea*), multiple paternity levels correlate with the abundance of individuals in the mating system, being much higher in arribada rookeries than in solitary nesting sites. We used two highly polymorphic microsatellite markers (Cm84 and Or1) to assess the level of multiple paternity in an olive ridley solitary population nesting in the Gulf of Fonseca, Honduras. We found evidence of multiple paternity in 6 out of 8 clutches (75%), with a minimum number of two fathers in four clutches, and a minimum of three in the remaining two clutches. This high level of multiple paternity in a small solitary population suggests that some of the females nesting in Honduras may be coming from proximal Nicaraguan arribada nesting beaches. Historical evidences and recent satellite telemetry data support this hypothesis. In addition, we show that multiple paternity studies can be effectively performed in the absence of maternal samples, and that pooled DNA samples can be used with results comparable to individual hatchling sampling in multiple paternity analyses.
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1. Introduction

In order to make effective management decisions and improve current conservation projects on nesting beaches, it is important to accurately estimate population size, population structure, and reproductive behavior. In populations where polyandry occurs, multiple paternity influences the effective population size (Sugg and Chesser, 1994) and the genetic variability within a population (Baer and Schmid-Hempel, 1999). Multiple paternity studies yield valuable information regarding mating patterns, and help in understanding population structure (Jensen et al., 2006). Recent studies have shown evidence of multiple paternity in all sea turtle species: green (*Chelonia midas*) (FitzSimmons, 1998; Lee and Hays, 2004), loggerhead (*Caretta caretta*) (Moore and Ball, 2002; Zbinden et al., 2007b), leatherback (*Dermochelys coriacea*) (Crim et al., 2002; Stewart and Dutton, 2011), hawksbill (*Eretmochelys imbricata*) (Joseph and Shaw, 2011), flatback (*Natator depressus*) (Theissinger et al., 2009), olive ridley (*Lepidochelys olivacea*) (Hoeckert et al., 1996; Jensen et al., 2006) and Kemp's ridley (*Lepidochelys kempi*) (Kichler et al., 1999), with high inter- and intraspecific variability (Uller and Olsson, 2008). In the case of olive ridleys, Jensen et al. (2006) showed that multiple paternity strongly depends on reproductive patterns, with arribada nesters showing much higher rates than solitary nesters. They suggested that the frequency of multiple paternity depends primarily on the abundance of individuals in the mating system, and calculated the relationship between population size and multiple paternity levels for the genus *Lepidochelys*.

Because of their abundance, high polymorphism content, codominance, easy detection, and transferability among studies, microsatellites are ideal molecular markers for paternity studies (Aggarwal et al., 2004). For assessing multiple paternity in sea turtle clutches, it is not unusual to analyze both the mother and the offspring. Once the maternal alleles for each microsatellite marker have been identified in the hatchlings, the remaining alleles constitute the paternal contribution. Multiple paternity can be inferred in egg clutches laid by a single mother from the presence of more than two paternal alleles, with three alleles meaning at least two fathers, and five alleles meaning at least three fathers. The analysis of individual offspring genotypes allows estimating the paternal contribution of each father, and in some cases, identifying actual multiple paternity with less than five total alleles, when the observed distribution of alleles is not compatible with only one father (Jensen et al., 2006).

Although determination of multiple paternity in single clutches based on knowledge of the maternal and individual offspring genotypes is the ideal and most informative procedure for multiple paternity studies, this protocol may sometimes be impractical or impossible. In some cases, samples from mothers may be unavailable, such as in

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conservation projects where beaches are monitored and nests are collected during morning patrols (de Santos Loureiro, 2008; Godgenger et al., 2009; Zbinden et al., 2007a). In other cases, the high number of samples required in typical multiple paternity studies (Hoeckert et al., 1996; Hoekert et al., 2002; Jensen et al., 2006; Stewart and Dutton, 2011), become cost prohibitive. Taking into account that it is the total number of different alleles in each clutch that establishes both presence and frequency of multiple paternity in a population, maternal samples are not strictly necessary for detecting multiple paternity. In the absence of female samples, the presence of five or more alleles in a single nest evidences at least two contributing males, and thus, multiple paternity. Pearse et al. (2002), Theissinger et al. (2009), and Valenzuela (2000) have previously applied this criterion in turtle multiple paternity studies that lacked maternal samples for a number of clutches. The specific genotypes of individual offspring are not essential information either. Given that multiple paternity can be correctly assessed knowing the total number of alleles in a clutch, it may be advantageous in terms of time and budget to obtain this information via pooled samples from hatchlings in a clutch, rather than by individually analyzing each hatchling sample. The use of pooled samples for microsatellite analysis has been successful in human forensic and epidemiological studies with high numbers of individuals (Pacek et al., 1993; Sham et al., 2002). However, due to the frequent presence of PCR artifacts, the electrophoretic patterns of pooled samples tend to be complex and difficult to interpret (Schnack et al., 2004). Preliminary DNA pooling is recommended for paternity studies, to reduce the number of tests required to identify potential parents for an individual progeny (Curnow and Morris, 1998), but is rarely used in multiple paternity studies. Gosselin et al. (2005) pooled eggs from individual pleopods in a multiple paternity study on the American lobster, *Homarus americanus*, following Urbani et al. (1998), who previously used this method with the snow crab, *Chionoecetes opilio*. To our knowledge, the use of pooled DNA samples has not been reported to date in multiple paternity studies on sea turtles or any other vertebrates.

Olive ridley sea turtle eggs have been economically exploited in the South coast of Honduras since the 1940s (Campbell, 2007). Significant population declines led to the implementation of conservation measures by the Honduran government in 1975, which established an ongoing yearly protected period when the collection of eggs is forbidden and the eggs are relocated to hatcheries (Minarik, 1985). However, studies on the olive ridley population nesting in Honduras are scarce. The environmental NGO *Protective Turtle Ecology Center for Training, Outreach, and Research, Inc.* (ProTECTOR) has been monitoring the nesting beaches since 2007 (Dunbar and Salinas, 2008; Dunbar et al., 2010), yet until now, no genetic studies have been performed.

Olive ridley nesting beaches in Honduras are located within the Gulf of Fonseca, a shallow-water inlet of the Pacific Ocean, 80 km long and 50 km wide, sheltered by islands at its entrance (Lemay et al., 2007) (Fig. 1). Punta Ratón (13.26570 N, 87.51228 W) is the main nesting beach in the country, with an estimated number of 400–500 nests per season (Dunbar, personal communication). Three other nesting beaches are known along the east coast of the Gulf: El Venado (13.11581 N, 087.42725 W), which receives approximately 200–250 nests per season; along with smaller sites at Boca del Río Viejo and Cedeño, with approximately 80–140 nests each per season (Dunbar, personal communication). According to historical reports, 100% of eggs from Punta Ratón were consumed for more than three decades (1940s–1970s) (Campbell, 2007) before the establishment of protection measures. The fact that the turtle population nevertheless persisted caused Pritchard (2007b) to speculate that females nesting at Punta Ratón may, in reality, come from arribada populations at the Chacocente and La Flor beaches in Nicaragua (Pritchard, 2007b).

The main goals of our study were: 1) to assess the levels of multiple paternity in the olive ridley sea turtle population nesting in the South

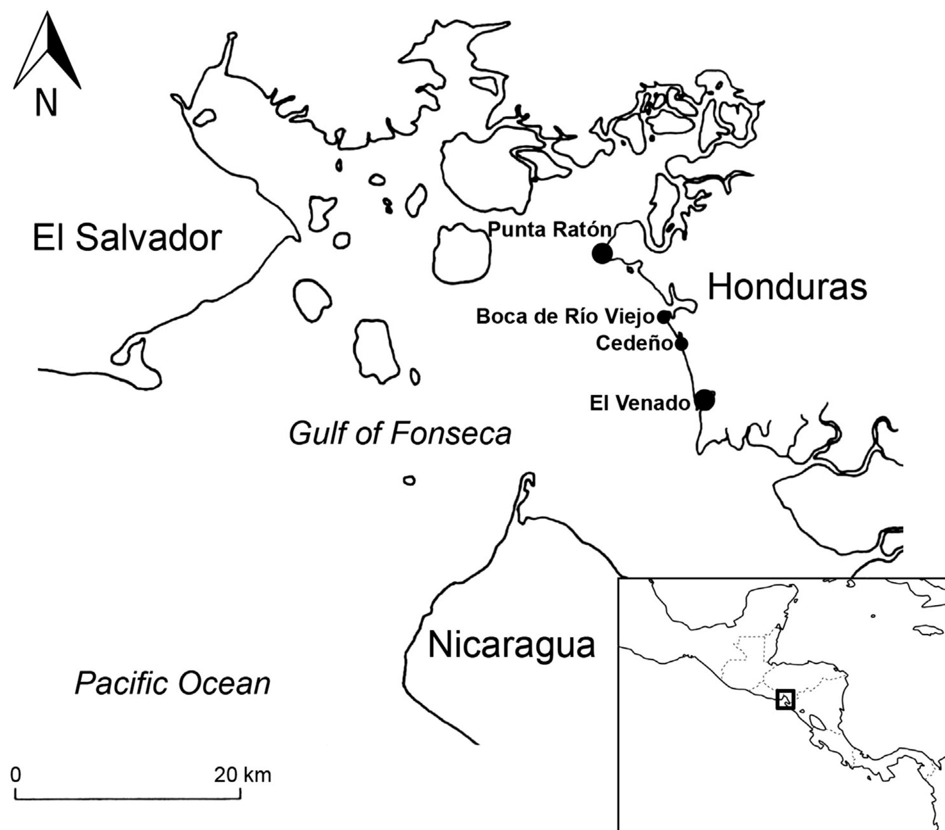

Fig. 1. The Gulf of Fonseca. Circles indicate the four main nesting beaches for *L. olivacea* on the South coast of Honduras. Large circles indicate the two main *L. olivacea* nesting beaches at Punta Ratón and El Venado, the field sites for this study.

coast of Honduras, and 2) to use levels of multiple paternity to estimate population size and origin of the nesting females present in the Gulf of Fonseca. Besides specific information about the Honduran olive ridley population, this study also allowed us to investigate new methods to determine sea turtle multiple paternity, with a potentially wider application. Two additional goals were 1) to confirm that multiple paternity studies can be effectively performed without female samples, and 2) to evaluate the potential of using one-per-clutch pooled samples to detect multiple paternity, rather than individually analyzing multiple hatchling samples.

2. Methods

2.1. Field sampling

During the 2011–2013 nesting seasons (August to November), we collected blood and tissue samples from 26 nesting females at Punta Raton and El Venado (Fig. 1). Blood samples (1–2 ml) from the dorsal cervical sinus were collected from 15 females at Punta Raton and 5 females at El Venado. In 6 cases we collected tissue samples from females nesting at Punta Ratón by cutting a small piece of skin (<25 mm^2) from the soft tissue of the posterior edge of the left rear flipper. We marked all sampled females with flipper tags on the left front flipper. Hatchlings from three nests of the 2012 season and six nests of the 2013 season were also sampled. Immediately after emergence, we randomly selected 20 hatchlings from each nest and took blood samples (<0.1 ml) from the dorsal cervical sinus. We monitored sampled hatchlings for 1 h after the procedure to ensure normal behavior, and released them as soon as possible after observance. Blood and tissue samples were stored at room temperature in cell lysis buffer [10 mM Ethylenediaminetetraacetic acid (EDTA), 2% sodium dodecyl sulfate (SDS), 10 mM Tris-base — 8.0 pH]. Samples from 2011 and 2012 were kept at room temperature until June of 2013, when they were imported to the US and stored at 4 °C in the laboratory. Samples from the 2013 season were imported to the US and stored at 4 °C in the laboratory in November, 2013. All samples were analyzed between July and December, 2013.

2.2. Microsatellite analysis

Total genomic DNA was extracted from tissue samples following Prager and Stoneking (1999). For DNA extraction from blood samples, a modification of the same protocol was used (Supplementary Table S1). DNA concentration was checked with a NanoDrop 2000c UV–vis Spectrophotometer (Thermo Scientific, MA) and adjusted to 50–100 ng/μl. Pooled samples for each nest were prepared by mixing 1 μl of each of the hatchling samples together and adjusting the final concentration to 50–100 ng/μl. The microsatellite primers Cm84 and Or-1 (Supplementary Table S2) were selected for the paternity analysis because they had shown high variability and effective amplification in previous studies with Eastern Pacific olive ridley populations (Jensen et al., 2006). Microsatellites were amplified with fluorescent-labeled primers in 50 μl PCR reactions containing 50–100 ng of nuclear DNA, 10 pmol forward 6FAM 5-end labeled primer, 10 pmol reverse unlabeled primer (Integrated DNA Technologies, IA), and 25 μl of Maxima Hot Start PCR Master Mix (Thermo Scientific, MA).

Thermal cycling was initiated with a 4 min denaturing step at 95 °C for both Cm84 and Or-1, followed by 35 cycles of 30 s denaturation at 95 °C (Cm84 and Or-1), 30 s annealing at 56 °C for Cm84 (55 °C for Or-1), and a 30 s (1 min for Or-1) extension at 72 °C for both Cm84 and Or-1, and a final extension of 5 and 10 min at 72 °C for Cm84 and Or-1, respectively. PCR products were run on a 5% polyacrylamide gel (Bio Rad, CA) at 60 W for 2 h to confirm DNA presence. Following PCR using template DNA from individual hatchlings, a separate pooled sample of the PCR products for each nest was prepared by mixing 1 μl of each PCR product from the nest. In a second PCR reaction, the pooled DNA mentioned in the previous paragraph was used as the template. The PCR products were sent to an external laboratory (Genewiz, NJ), where fragment analysis was performed on an ABI3730 DNA Analyzer (Life Technologies, CA). PCR products from 17 to 20 hatchlings were analyzed from each of 9 nests totaling 158 individuals, plus two pooled samples for each nest, one pooled before PCR and one pooled after PCR. Maternal samples were only available for the six 2013 season nests, yet only three of them yielded useful PCR products. In these cases, products from adult females were run along with the products from their offspring. In addition, 26 samples from non-related adult females and 6 hatchlings from different nests were analyzed to assess population diversity. Results from the DNA Analyzer were visualized using Geneious 6.1.7 created by Biomatters.

2.3. Data analysis

2.3.1. Population analysis

PCR products from 32 individuals were analyzed to estimate the allele frequencies for Cm84 and Or1 in the population, yet only 27 genotypes were obtained, corresponding to 15 nesting females from Punta Raton, 6 nesting females from El Venado and 6 hatchlings randomly selected from nests with no maternal samples (one hatchling per nest). We assumed that all the sampled animals were unrelated. Data was checked for departure from Hardy–Weinberg equilibrium, genotypic linkage disequilibrium, and the presence of null alleles using GENEPOP 4.2.2 (Rousset, 2008).

2.3.2. Paternity analysis

For those nests with known maternal genotypes, paternal alleles were inferred from offspring genotypes once maternal alleles were accounted for, and confirmed using GERUD 2.0 (Jones, 2005). For nests with no maternal samples, offspring genotypes were directly analyzed with GERUD 2.0 to determine all possible maternal genotypes and the corresponding paternal genotypes for each case. We also used GERUD 2.0 to calculate exclusion probabilities. To calculate the probability of detecting multiple paternity with unknown parents for the number of offspring sampled in the study we used the PrDM software (Neff and Pitcher, 2002) and GERUDsim 2.0 (Jones, 2005). GERUDsim 2.0 uses a simulation approach to determine the ability of GERUD 2.0 to correctly determine the number and genotype of sires for specific progeny.

We confirmed paternity results obtained with GERUD 2.0 using COLONY (Wang, 2004). Because we sought to assess the minimum number of sires required to explain offspring genotypes (MIN estimates), we used the MIN method from Sefc and Koblmüller (2009), COLONY calculations include the possibility of two error classes: null alleles (Class I), and typing errors and mutations (Class II). We used error rates of 0.05 for both classes (Wang, 2004).

GERUD 2.0 and COLONY estimates for multiple paternity were obtained from the analysis of individual samples. Results from pooled samples were visually analyzed using Geneious 6.1.7, and the sizes and total number of alleles present in each clutch were compared with those obtained from the individual samples of the same clutch. For samples that contained high levels of unresolved peaks, we used the program Poolfitter v1.1 (Schnack et al., 2004) to remove stutter noise and identify the true allelic peaks (Fig. 2). When interpreting Poolfitter outcomes, we removed peaks lower than 0.1 of the total frequency and consider the remaining peaks to be true alleles.

3. Results

3.1. Population variability

Both loci were highly polymorphic, with 16 and 13 alleles found at microsatellites Cm84 and Or1, respectively. Although Cm84 is a dinucleotide repeat, several alleles for this locus differed by only one bp, a fact previously observed in other studies (Hoekert et al., 2002). Expected

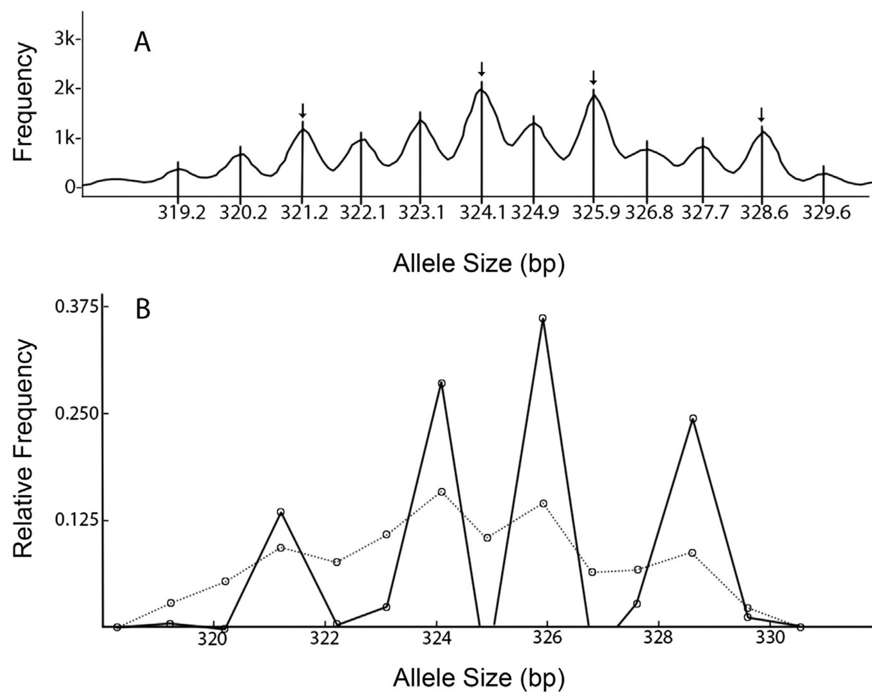

Fig. 2. Analysis of Cm84 pooled samples. (A) Electrophoretic pattern of nest H1 from Geneious 6.1.7, showing a high number of stutter peaks almost indistinguishable from the true allelic peaks (shown with arrows). (B) Stutter correction with Poolfitter v1.1. Dotted line shows the original pattern and solid line shows the corrected pattern. Individual peaks are represented as circles. X axis shows allele sizes, and Y axis shows relative frequencies.

heterozygosities were 0.89 for Cm84 and 0.85 for Or1. Observed heterozygosities were slightly lower for Cm84 (0.83) and slightly higher for Or1 (0.92). No loci exhibited significant departure from Hardy–Weinberg equilibrium ($P > 0.05$), and no linkage disequilibrium was detected between loci ($P > 0.05$). The test for null alleles showed possible null alleles at very low frequency for one locus (Cm84; 0.0471), likely caused by scoring errors (Jensen et al., 2006).

3.2. Multiple paternity

3.2.1. Individual samples

Multiple paternity was inferred when the total number of alleles per locus was 5 or more, and confirmed by GERUD 2.0 outcomes. One case with less than 5 alleles at one locus was also identified as resulting from multiple paternity when the distribution of alleles across loci could not be attributed to only one father. We considered alleles present in just one locus and only one offspring likely resulting from mutation events or scoring errors (Jensen et al., 2006). After removing the corresponding hatchlings from the analysis, the sample size of offspring analyzed per clutch ranged from 12 to 19, with a mean of 16 ± 1 SE. The highest number of alleles in a single clutch was seven and the highest minimum number of fathers identified by GERUD 2.0 was three (Table 1). Only three maternal samples yielded DNA and were used in the analysis. For each of the three nests with known maternal genotypes (B1, B3 and H26), results from GERUD 2.0 on multiple paternity and minimum number of fathers were the same, whether or not maternal samples were included in the analysis. Among the nine clutches studied, one (B2) yielded results incompatible with only one mother, probably due to contamination. From the eight remaining clutches, two (25%) showed no evidence of multiple paternity, while four (50%) had a minimum of two fathers, and two (25%) had a minimum of three fathers (Table 1). The two clutches sired by a single father (B3 and N38) showed low variability. Among the offspring in clutch B3, three different alleles were found at Cm84 and only two at Or1 (Table 2). The female was homozygous at Cm84 (326/326) and both parents shared the same genotype at Or1 (148/168). In clutch N38, we found only three different alleles at each locus. Not having a maternal sample for this clutch, the software GERUD 2.0 was unable to yield a single solution for maternal and paternal genotypes. However, in all four possible combinations, parents shared one allele at Cm84 (325) and either also shared one allele at Or1, or one of the parents was homozygous (Table 2).

With the loci and sample sizes used, the combined exclusion probability was 0.92 for the clutches with one known parent, and 0.81 for those with neither parent known. The probability of detecting multiple paternity (PrDM) assuming equal paternal contributions was high when sampling 10 offspring (PrDM = 0.96) and only slightly higher when sampling 15 (PrDM = 0.98). A skewed paternal contribution of 1:4 would still give a PrDM > 0.9 when sampling 15 offspring. However, a very skewed case of 1:9 would reduce the PrDM to 0.72.

Simulation analyses with GERUDsim 2.0 for an offspring of 15 indicated that multiple paternity would be detected in 97.1% of clutches

Table 1
Multiple paternity results. The table shows the number of hatchlings originally analyzed in each nest and the final number after removing the hatchlings with just one 'extra' paternal allele at one locus. The number of different alleles at the microsatellite loci Cm84 and Or1 is given, both the total found and the final number after removing probable mutations and scoring errors. The minimum number of fathers inferred by the program GERUD 2.0 was calculated using the final values. Because COLONY incorporates error rates in its calculations, the minimum number of fathers inferred by COLONY was calculated using the whole data.

Nest	No. of hatchlings analyzed (final no.)	Cm84 alleles (final no.)	Or1 alleles (final no.)	Minimum number of fathers GERUD 2.0	Minimum number of fathers COLONY
B1	19 (18)	7 (6)	7	3	4
B2	17	7	7	No results[a]	4
B3	19	3	2	1	1
H1	18 (17)	6 (5)	4	2	2
H2	17	4	7	3	3
H26	18 (12)	9 (5)	6 (5)	2	2
N37	16 (14)	4 (2)	5 (4)	2	2
N38	18	3	3	1	1
N40	16 (13)	7 (5)	6 (5)	2	3

[a] Nest B2 results were not compatible with just one mother, probably due to contamination. This nest was removed from the study.

Table 2
Genotypes of nests sired by only one male (B3 and N38).

(A) Nest B3 genotypes. Maternal and offspring genotypes were obtained from samples, and the paternal genotype was inferred by GERUD 2.0

Maternal genotype		Number of offspring	Offspring genotypes		Genotype frequency	Inferred paternal genotype	
Cm84	Or1		Cm84	Or1		Cm84	Or1
326/326	148/168	19	326/337	168/168	2	322/337	148/168
			326/322	148/168	4		
			326/337	148/168	4		
			326/337	148/148	3		
			326/322	168/168	3		
			326/322	148/148	3		

(B) Nest N38 genotypes. Offspring genotypes were obtained from samples. Parental genotypes were inferred by GERUD 2.0. The software was unable to identify one single pair of mother–father genotypes, but found four different possible combinations.

Number of offspring	Offspring genotypes		Genotype frequency	Inferred genotypes			
				Parent 1		Parent 2	
	Cm84	Or1		Cm84	Or1	Cm84	Or1
18	325/325	152/168	4	325/341	148/168	325/344	152/152
	341/344	152/168	3				
	325/344	148/152	1	325/341	148/152	325/344	152/168
	325/344	152/168	2				
	325/341	152/168	2	325/341	148/152	325/344	148/168
	325/341	148/152	3				
	341/344	148/152	3	325/344	152/168	325/341	148/168

for equal contributions (8:7) and in 91.4% in case of a very skewed paternal contribution (13:2).

Paternity reconstructions using COLONY confirmed GERUD 2.0 outcomes. Because COLONY reconstructions already take into account both mutations and typing errors, as well as null alleles, we used the entire data set, without removing the extra alleles that appeared in just one locus in a single hatchling. COLONY found multiple paternity in the same 6 nests as the GERUD 2.0 analysis, although the minimum estimated number of fathers was higher in two nests (Table 1). The number of hatchlings analyzed ranged from 16 to 19. Two nests (25%) were sired by only one male and 6 nests (75%) showed multiple paternity, with a minimum of two fathers in three nests, a minimum of three fathers in two nests, and a minimum of four fathers in one nest. Because COLONY analysis includes the possibility of errors in the data, this program could make a parental reconstruction of nest B2, which GERUD 2.0 considered incompatible with only one mother. According to COLONY, this clutch was sired by at least 4 fathers. COLONY tends to overestimate the number of parents when the number of loci analyzed is low (Jones et al., 2007; Sefc and Koblmüller, 2009), while GERUD 2.0 is considered more accurate when paternity analyses can be run individually with less than 6 sires (Jones, 2005). For this reason, we chose to use COLONY results only for confirmation of our GERUD 2.0 outcomes, and excluded nest B2 from the analysis.

3.2.2. Pooled samples

Both Or1 and Cm84 pooled samples showed stutter peaks, although Or1 could be resolved by visual comparison with a few individual samples. Cm84 were corrected using Poolfitter v1.1.

Alleles detected in the Or1 pooled samples were the same in both pooled samples for each nest, before and after PCR (Fig. 3), and

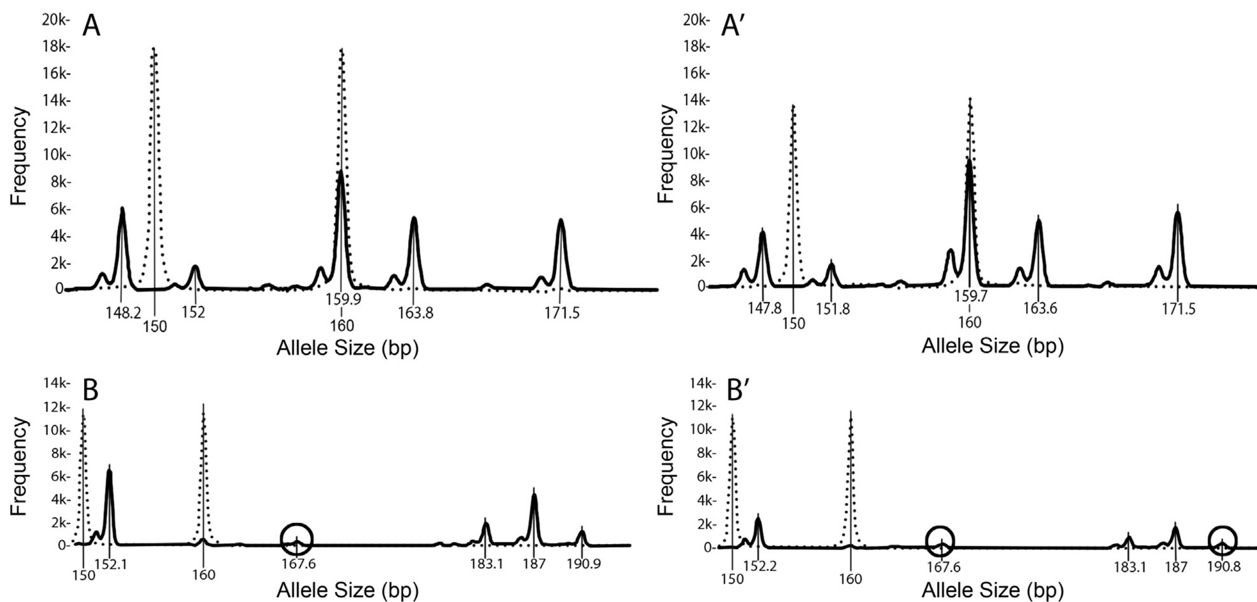

Fig. 3. Examples of alleles identified in Or1 pooled samples, visualized with Geneious 6.1.7. We show the graph for nests N37 (A, top) and H26 (B, bottom), both for samples pooled before PCR (A, B; left), and pooled PCR products (A′, B′; right). Solid line peaks indicate different alleles present in the nest, and dotted line peaks represent reference scale sizes. Numbers indicate allele sizes in bp. Y axis shows frequencies in relative fluorescence units. Small peaks that may be indistinguishable from noise are circled.

coincided with the alleles identified through individual analyses (Table 3). For some alleles with very low frequencies, such as those present in only one hatchling, the peaks were small and could easily be confused with noise (Fig. 3B and B′). After removing these small peaks, the total number of alleles detected in the combined samples was lower than the number obtained from the analysis of the individual samples in 3 out of 8 clutches (37.5%). In two clutches (B1 and H26) the sample pooled before PCR was more informative than the one pooled after PCR, revealing one additional allele (Fig. 3B, Table 3). When a total of 5 or more alleles was used as an indicator of multiple paternity, and results from pooled and individual samples were compared, results from both approaches coincided in 6 clutches (75%) (Table 3). The remaining two clutches, H26 and N40, showed evidence of multiple paternity when the samples were individually analyzed, yet only 4 different alleles appeared in the pooled samples (Table 3). Clutch H1 showed only 4 alleles both in the individual and pooled analyses, not allowing multiple paternity to be inferred. However, the distribution of alleles in the individual samples was not compatible with only one father. Two hatchlings from this clutch were homozygous for allele 152, meaning that the mother and the father shared this allele, increasing the minimum number of paternal alleles to three. This fact, along with information from locus Cm84, strongly suggested multiple paternity for this clutch.

Because Or1 samples pooled before PCR gave better results than pooled PCR products, we only used the Cm84 samples pooled before PCR. After stutter correction with Poolfitter, the number of alleles detected in the Cm84 pooled samples was lower than the higher number found through individual analysis in five cases (62.5%), equal in two (25%), and higher in one (12.5%) (Table 3). In spite of these differences, pooled samples gave us the same information as individual samples regarding multiple paternity in 6 out of 8 clutches (75%). Under both approaches, nests B1 and N40 contained 5 or more different alleles, indicating more than one sire, whereas nests B3, N38, H2 and N37 showed 4 or fewer alleles, not allowing the inference of multiple paternity. In the remaining two nests (25%), H1 and H26, the pooled sample did not indicate evidence of multiple paternity, while the analysis of individual samples did.

Adding the outcomes of the analysis of both Or1 and Cm84 pooled samples, we obtained evidence of multiple paternity for 4 out of 8 nests (B1, H2, N37 and N40), whereas 4 nests (B3, H1, H26 and N38) appeared to be sired by only one male each. These results coincided with the results obtained from the analyses of individual samples in six cases (75%). Nests H1 and H26 showed evidence of multiple sires when samples were analyzed individually, although multiple paternity was not detected in any of the pooled samples from these nests.

4. Discussion

This study documents the presence of high levels of multiple paternity in the olive ridley population nesting within the Gulf of Fonseca, Honduras. Although sample size (8 clutches), as well as the number of hatchlings analyzed per clutch (12–19) were relatively low, multiple paternity was unambiguously found in 75% of the nests, with a minimum of two contributing males in 66% of them, and a minimum of three contributing males in the remaining 33%. In the interpretation of results, we used a conservative approach, meaning that both the level of multiple paternity in the Honduran olive ridley population and the minimum number of fathers per clutch may actually be higher than detected.

Even if slightly underestimated, multiple paternity levels detected here fell within the range demonstrated in previous studies. Hoekert et al. (2002) found 20% multiple paternity in the olive ridley population nesting in Surinam and, in a study on the Pacific coast of Costa Rica, Jensen et al. (2006) detected highly different levels of multiple paternity between a solitary population nesting at Playa Hermosa (30%) and the arribada population nesting at Ostional (92%). These two geographically close populations showed no evident genetic differentiation, suggesting that differences in multiple paternity levels among olive ridley populations may well be caused by extrinsic factors rather than being a genetically determined phenomenon (Jensen et al., 2006). The authors concluded that the abundance of individuals in the mating system was the dominating factor related to multiple paternity.

Although several benefits have been proposed to explain the evolution and selection of polyandry and multiple paternity (Uller and Olsson, 2008; Zeh and Zeh, 2001), no direct benefits have been demonstrated to date for sea turtle females. In fact, studies on green turtles (Lee and Hays, 2004; Wright et al., 2013) highlighted a potential cost to polyandry, and suggested that females mate several times in response to male coercion to avoid the potentially higher costs of aggressive male harassment. The advantages of multiple mating for males are obvious and thus it is likely that polyandry and multiple paternity in sea turtles are actually driven by the benefits to males mating as often as possible with as many females as possible (Lee and Hays, 2004). Due to the ease of finding females for successive mating encounters, large aggregations with high densities of mature individuals off arribada rookeries provide optimal mating opportunities for males, giving rise to the potential for high levels of multiple paternity. However, in a solitary population with a relatively small offshore aggregation, expected multiple paternity levels are likely to be much lower. A trend in increasing multiple paternity with increasing female breeding population size has been reported by Ireland et al. (2003) using data from different sea turtle species. Jensen et al. (2006) detected the same trend when

Table 3
Pooled samples results. Number of Or1 and Cm84 parental alleles from individual and pooled samples, along with accuracy of results from pooled samples to correctly detect multiple paternity.

Nest	Or1				Cm84			Correct multiple paternity detection (Or1 & Cm 84)
	No. of alleles from individual samples (final no. after removing possible mutations)	Total no. of alleles from samples pooled before PCR	Total no. of alleles from samples pooled after PCR	Correct multiple paternity detection	No. of alleles from individual samples (final no. after removing possible mutations)	Total no. of alleles from samples pooled before PCR	Correct multiple paternity detection	
B1	7	6	5	Yes	7 (6)	8	Yes	Yes
B3	2	2	2	Yes	3	3	Yes	Yes
H1	4	4	4	No[a]	6 (5)	4	No	No
H2	7	7	7	Yes	4	3	No[b]	Yes
H26	6 (5)	4	3	No	9 (5)	4	No	No
N37	5 (4)	5	5	Yes	4 (2)	3	No[b]	Yes
N38	3	3	–	Yes	3	3	Yes	Yes
N40	6 (5)	4	4	No	7 (5)	5	Yes	Yes

[a] The analysis of the pooled sample for this nest assessed the correct number of alleles (4), but the distribution pattern of the alleles in the individual samples indicated the presence of two fathers.

[b] Information from Cm84 for nests H2 and N37 did not allow to infer multiple paternity because the total number of alleles was less than 5 both in the individual sample and in the pooled sample analysis. However, information from Or1 individual samples revealed multiple paternity in both nests.

plotting multiple paternity data from ten rookeries against breeding population size. When taking into account only data for the genus *Lepidochelys*, they found a significant fit to an exponential regression (Fig. 4).

Recent multiple paternity studies confirmed the general trend of higher levels of multiple paternity associated with larger populations (Fig. 4). Joseph and Shaw (2011) found 20% multiple paternity in a small population of hawksbill sea turtles from Sabah Turtle Islands, Malaysia, whereas a several fold times larger population of green turtles from the same area showed multiple paternity levels of 71% (Joseph, 2006). However, some discordant data have also appeared (Fig. 4). A study on flatbacks from Queensland, Australia, by Theissinger et al. (2009), showed multiple paternity in 69% of the clutches in a population with an estimated size of only 2650 adult females. Zbinden et al. (2007b) reported the highest multiple paternity rate found in a sea turtle population (93%) in the Mediterranean loggerhead rookery of Zakynthos, Greece, with an estimated breeding population size of less than 500 females. Additionally, Lasala et al. (2013) studied a small loggerhead nesting beach on Wassaw Island, Georgia, and found multiple paternity in 75% of the clutches although the population size did not exceeded 200 females. One suggested explanation for finding extremely high rates of multiple paternity in small populations is that the most influencing factor is not the actual number of animals present, but their density within the mating area (Zbinden et al., 2007b). In the Zakynthos rookery, turtles aggregate in a narrow area of the Bay of Laganas, 9 km long by 1 km wide, where densities may reach 54 individuals/km^2 (Schofield et al., 2009). In Georgia, the ocean floor off Wassaw Island drops sharply in a feature known as the Georgia Bight, which may limit the size of the area for loggerheads to congregate, also resulting in a dense concentration of turtles (Lasala et al., 2013).

Our current study on Honduran olive ridleys has also yielded anomalously high rates of multiple paternity. When we use Jensen's regression graph (Jensen et al., 2006) to calculate the breeding population size corresponding to the multiple paternity level found in the current study (75%), the result approaches 40,000 individuals (Fig. 4). Although information on this population is scarce, data from beach monitoring compiled by ProTECTOR during the last seven years strongly suggests that the actual population size is approximately two orders of magnitude lower than this figure. Female breeding population size can be estimated as the total number of clutches laid in the population, divided by the mean clutch frequency, adjusted by the estimated remigration interval (Ireland et al., 2003). One thousand appears to be a generous estimate of the number of nests deposited annually along all nesting beaches in the South coast of Honduras (Dunbar, personal communication). Solitary olive ridleys lay two to three clutches per year (Miller, 1997), with a mean interesting interval of 14 days, and tend to nest annually (Plotkin, 1994; Pritchard, 1969). From these data we estimate a breeding population size of 333 to 500 females. Even if the number of nests is doubled to 2000 (supposing that many nests may remain undetected on non-monitored beaches), and the less common remigration interval of 2 years is used, the estimated breeding population size would be 1333 to 2000 females, still far short of the calculated 40,000 using Jensen's regression (Fig. 4).

Although the density of animals in the breeding area has not been calculated, high density is not likely to be the best explanation for increased multiple paternity rates in this case. The Gulf of Fonseca is an important area for artisanal fishing in Honduras (Dunbar et al., 2012), and more than 300 small boats fish daily in the Honduran waters of the gulf, covering most of its area (Box and Bonilla, 2009). If the sea turtle density was as high as those reported in Costa Rica mass nesting beaches, or in the Zakynthos rookery in Greece, fishers should be reporting sea turtle encounters very frequently during the nesting season. Yet, reported sea turtle sightings are only occasional (Dunbar, personal communication). In this case, behavioral information specific to olive ridleys may help explain the observed discrepancy between the expected and observed multiple paternity levels. Cheloniids

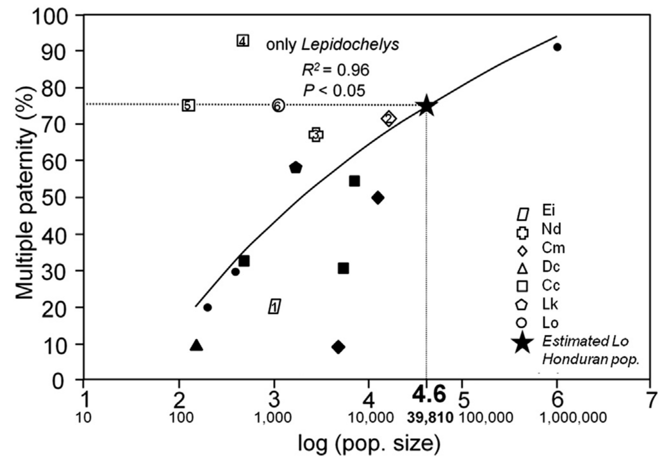

Fig. 4. Estimated size of the Honduran *L. olivacea* population (indicated by a star) derived using the exponential regression graph from Jensen et al. (2006). Solid symbols indicate data from the original graph by Jensen et al. (2006). Open symbols indicate data from recent studies: (1) Joseph and Shaw (2011), (2) Joseph (2006), (3) Theissinger et al. (2009), (4) Zbinden et al. (2007a, 2007b), (5) Lasala et al. (2013) and (6) this study. When the study did not report data on adult female population size, an estimate was calculated as the total number of clutches divided by the mean clutch frequency, adjusted by the estimated remigration interval. Ei: *Eretmochelys imbricata*, Nd: *Natator depressus*, Cm: *Chelonia midas*, Dc: *Dermochelys coriacea*, Cc: *Caretta caretta*, Lk: *Lepidochelys kempi*, Lo: *Lepidochelys olivacea*.

(hard-shelled sea turtles) are known for their high fidelity, both to feeding areas (Broderick et al., 2007) and to nesting beaches (Plotkin, 2003). Olive ridleys, however, lack the highly specialized life history stages characteristic of the other cheloniid species (Pritchard, 2007a), showing high behavioral plasticity and adaptability to variable environmental factors. Recent studies of the Eastern Pacific population found that *L. olivacea* do not follow specific migratory corridors, do not show site fidelity to feeding areas, and change their movement patterns in response to climatic events, such as El Niño Southern Oscillation (ENSO) (Plotkin, 2010). Nest site fidelity tends to be low for *L. olivacea* solitary nesters (Kalb, 1999), which may switch between beaches separated by dozens to hundreds of kilometers within a single nesting season (Schulz, 1971; Tripathy and Pandav, 2008). Arribada nesters generally show high site fidelity and remain close to the nesting beach during the entire season (Pandav et al., 2000), yet reports of arribada females nesting alternately between different rookeries are not uncommon (Cornelius and Robinson, 1985; Pandav, 2000; Tripathy and Pandav, 2008). Some females are also known to use a mixed strategy, switching between mass and solitary nesting, even during a single season (Bernardo and Plotkin, 2007; Kalb, 1999). Low nest site fidelity along with the possibility of females moving back and forth from mass nesting to solitary beaches may explain the disparity of results we obtained when estimating the size of the Honduran olive ridley population.

If the Gulf of Fonseca population is not discrete, but contains a number of females coming from proximal arribada beaches, multiple paternity levels above normal values for a small solitary population may be expected. Pritchard (2007b), who worked on the beach at Punta Ratón during the 1960s and recorded 100% egg collection and consumption by community members, also proposed this hypothesis. Essentially, complete egg collection occurred from at least the 1940s and until 1975, when the Honduran Government established the first "veda" protected period. Although the population should have been extirpated from the region, nesting females continued emerging to nest in Punta Ratón during the ensuing decades. Pritchard (2007a) suggested that instead of being returning hatchlings from Honduran nesting beaches, these females were probably coming from Nicaraguan mass nesting beaches. The closest mass nesting beaches to Honduras are Chacocente and La Flor, located approximately 220 km and 290 km south, respectively, from the mouth of the Gulf of Fonseca, and both

are within the distance range of observed inter-beach movements for olive ridleys (Tripathy and Pandav, 2008). Further support for the presence of external nesting females in the Gulf of Fonseca comes from recent satellite telemetry that showed a nesting female satellite tagged at El Venado, Honduras, leaving the Gulf and moving South along the coast of Nicaragua to subsequently reach the shore, presumably in an attempt to nest (Dunbar and Salinas, 2013). Beach exchange is a complex strategy that olive ridleys may use to colonize new areas or new beaches (Tripathy and Pandav, 2008), or to distribute the reproductive resources between arribada and solitary beaches to increase offspring survival. In any case, results of this study, along with recent satellite telemetry and historical data on the Honduran olive ridley population suggest that at least a number of the females nesting within the Gulf of Fonseca may be coming from close proximity arribada beaches, with Chacocente or La Flor in Nicaragua being likely candidates. Further research will be needed to confirm this hypothesis. Although genetic analysis of mitochondrial DNA has been previously used to identify the origin of sea turtles found in feeding grounds (Bowen et al., 1996; Sears et al., 1995) and to establish phylogeography and population structure of a sea turtle species in specific regions (Encalada et al., 1996), we do not recommend this approach due to the low levels of genetic differentiation that Eastern Pacific olive ridleys show between populations (Briseño-Dueñas, 1998; López-Chávez, 2000). Satellite telemetry may likely be a better option for confirming that females are moving from mass nesting beaches in Nicaragua to the Honduran waters within the Gulf of Fonseca.

Regarding the methodological goals of this study, we have shown that it is possible to perform successful multiple paternity studies in the absence of female samples. While the availability of maternal samples reduces uncertainty regarding the exact genotype of males and allows calculation of paternal contributions, in cases where minimal access to females is possible, such studies can still provide accurate estimates of multiple paternity levels in the population, as well as of the number of contributing males per clutch. Likewise, the use of pooled samples has the potential to be a valuable tool for sea turtle multiple paternity research, at least for obtaining fast and relatively inexpensive preliminary results. Although the analysis of pooled samples was less informative than the analysis of individual samples, the same alleles found in individual samples appeared in pooled samples and the presence or absence of multiple paternity was correctly revealed in 75% of clutches when pooled samples for both markers were used. Underestimation in pooled samples may occur in the case of alleles with very low frequencies, or when one or both parents is homozygous. However, both issues might be addressed with the use of several microsatellite markers. In cases where the presence of stutter PCR artifacts makes difficult to identify true allelic peaks, we suggest using specific software for stutter correction, such as Poolfitter (Schnack et al., 2004). Even if stutter correction is not needed, we strongly recommend analyzing at least some individual samples, and visually identifying the shape of the peaks for each particular locus. This will help distinguish true peaks from artifacts in an electropherogram from a pooled sample. Because pooling samples after DNA extraction yields slightly better results than pooling the PCR products, we also recommend pooling DNA samples of equal concentration before using the pooled sample as the template in PCR.

Multiple paternity studies are pivotal in sea turtle research. They provide information about reproductive behavior that it is difficult to observe directly. Multiple paternity levels impact effective population size and diversity, and thus need to be considered for management and conservation purposes. In the current study, high multiple paternity levels found in Honduran olive ridleys imply that the effective population size and diversity are likely higher than expected for this population. The results of this study also inform our understanding of possible origins of the nesting females outside the Gulf of Fonseca. Further studies are needed to confirm this interpretation. To know the origin, migration patterns, and reproductive behavior of Honduran nesting olive ridley turtles will help in designing more effective conservation plans for the population, as well as informing international cooperative management strategies for this species.

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