Reproductive success of male Atlantic spotted dolphins (*Stenella frontalis*) revealed by noninvasive genetic analysis of paternity

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Abstract: Cetaceans are known to frequently engage in sexual behavior; however, the lack of male parental investment in offspring makes assessment of male reproductive success difficult. We assessed paternity in a small population (mean individuals sighted per year = 93) of Atlantic spotted dolphins (*Stenella frontalis* (G. Cuvier, 1829)) utilizing noninvasively collected fecal material. Samples (n = 88) were collected from dolphins from four social clusters. Of the 29 offspring tested, 34.5% were assigned paternity, resulting in 10 paternities assigned to seven males. Our study indicates that achieving a certain age is a potential precursor for males to mate successfully, as 18 years was the youngest estimated age of a male at the time of calf conception. In all pairings but one, the males were older than the female (mean age difference = 7.7+ years). Successful males were from two of the four social clusters and males most often mated within their social group or with females from the next geographically closest group. The study combines genetic data with known maternal pedigree information and reveals patterns in the overall mating system in a cetacean species where reproductive success of males was previously unknown.

Résumé : Les cétacés participent fréquemment à des comportements sexuels, cependant l'absence d'investissement paternel envers les jeunes rend difficile l'évaluation du succès reproductif des mâles. Nous avons déterminé les paternités dans une petite population (nombre moyen d'individus aperçus par année = 93) de dauphins tachetés de l'Atlantique (*Stenella frontalis* (G. Cuvier, 1829)) par des récoltes de matériel fécal, une technique non invasive. Les échantillons (n = 88) proviennent de quatre regroupements sociaux. Nous avons déterminé la paternité de 34,5 % des 29 jeunes testés, obtenant ainsi dix paternités attribuées à sept mâles. Notre étude révèle que l'atteinte d'un certain âge est une condition potentielle pour l'accouplement réussi chez les mâles, puisque l'âge estimé le plus jeune d'un mâle à la conception d'un petit est de 18 ans. Dans tous les accouplements sauf un, le mâle est plus âgé que la femelle (différence d'âge moyenne = 7,7+ ans). Les mâles qui ont réussi appartiennent à deux des quatre regroupements sociaux et les mâles s'accouplement le plus souvent au sein de leur groupe social ou avec des femelles du groupe le plus rapproché géographiquement. Notre étude combine des données génétiques et des données d'ascendance maternelle et met en lumière des patrons dans le système global d'accouplement chez une espèce de cétacé dont le succès reproductif des mâles était encore inconnu.

[Traduit par la Rédaction]

Introduction

Knowledge of genetic relatedness is key to addressing questions about behavior and conservation in natural populations. Pedigrees provide the most complete picture of relatedness in a population and assessing paternity is an essential step in pedigree construction. As molecular techniques have advanced, studies of paternity in free-ranging terrestrial species have increased, but only a handful of studies investigating paternity in cetacean species are currently available. The disparity in the number of terrestrial and marine studies can certainly be attributed to the unique challenge of collecting genetic samples from species that spend only a fraction of their time at the ocean surface where genetic sample collection is most likely. Paternity studies in general present another challenge in that the number of paternities assigned and the statistical confidence with which those assignments are made increases if both the mother and the calf are sampled, along with a high proportion of the candidate fathers in the population. However, collecting genetic samples from known mother–calf pairs and candidate fathers is time consuming and field intensive. Therefore, not only is sample collection difficult, but researchers must also sample a relatively high proportion of the reproductive population to successfully assign paternity. Given the challenges, it is not surprising that paternity studies of cetaceans are infrequent.

Although rare, paternity research has already provided substantial information with respect to the behavioral and functional aspects of cetacean mating systems. An initial investigation into mating systems of humpback whales

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(*Megaptera novaeangliae* (Borowski, 1781)) reported a pattern of serial promiscuity in female whales, revealing that females mate with a different male each time that they are reproductively receptive (Clapham and Palsbøll 1997). Genetic examination of the breeding population of humpback whales in the West Indies showed socially dominant males achieved higher reproductive success than subdominant males (Nielsen et al. 2001). Among humpback whales breeding in the Mexican Pacific, male mating success was not random (Cerchio et al. 2005), but rather there was evidence of bias in male reproductive success. Mating skew, although evident, was not severe among identified fathers, indicating that many males are likely to contribute to subsequent generations even though some males have higher reproductive success. In North Atlantic right whales (*Eubalaena glacialis* (Müller, 1776)), Frasier et al. (2007) also found that male reproductive success was not random. Among the study group, there was significant reproductive bias towards older males. It was estimated that males did not achieve their first paternity until they reached 15 years of age, almost twice the age of first reproduction for females (Frasier et al. 2007).

Paternity research among toothed whales is even more limited. Amos et al. (1993) tested paternity of 34 fetuses of long-finned pilot whale (*Globicephala melas* (Traill, 1809)). For 33 of the 34 fetuses, the males within the pod could be excluded as the possible father. The results showed that although male pilot whales stay with their natal group, they do not mate within the pod. Paternity analysis of bottlenose dolphins (*Tursiops truncatus* (Montagu, 1821)) in Shark Bay, Australia, revealed that male reproductive success was significantly skewed toward members of long-term, stable associations of two or three males (Krützen et al. 2004). Although alliances likely serve multiple purposes, it was found that males in the alliances were more likely to successfully mate with females versus unpaired males (Krützen et al. 2004). Interestingly, Krützen et al. (2004) also reported an incestuous mating and a paternity assigned to a juvenile male bottlenose.

In this paper, we expand the current body of cetacean paternity research by focusing on the reproductive success of male Atlantic spotted dolphins (*Stenella frontalis* (G. Cuvier, 1829)) in the Bahamas. Atlantic spotted dolphins display the four developmental color phases originally described by Perrin (1970) for the pantropical spotted dolphin (*Stenella attenuata* (Gray, 1846)). The color phases were adapted for Atlantic spotted dolphins by Herzing (1997) and are a useful tool in estimating the age of an individual. Although there is some degree of individual variation, Atlantic spotted dolphins generally gain spots in a predictable manner throughout their development. Calves or "twotones" are born without spots and develop dark spots on the ventral surface when they are about 3 years of age when they enter the "speckled" age class. At roughly 9 years of age, ventral spotting increases and white spots develop on the dorsal surface and the individuals enter the "mottled" age category. Individuals in the "fused" age class consist of animals whose spots are no longer individually discrete but "fuse" together into an overall coloration pattern. The fused class is the oldest category and most individuals advance to this stage at approximately 16 years.

Female Atlantic spotted dolphins reach sexual maturity at approximately 8–11 years (Herzing 1997). The age of sexual maturity for male Atlantic spotted dolphins is currently unknown, but male pantropical spotted dolphins are reported to reach sexual maturity near 12–15 years (Perrin 2001). Gestation for Atlantic spotted dolphins lasts from 11 months to 1 year (Herzing 1997). The mean calving interval is 3 years but shortens when a female has lost a calf (Herzing 1997). Maternity is inferred from observed close associations between a female and a calf, including swimming in the echelon position, lactation, and nursing during the first years of life. The study population exhibits peak calving periods in early spring and late fall (Herzing 1997), although mating behavior has been observed throughout the entire field season (May–September). Mating and courtship behavior has been observed during all stages of development and between all age-class categories (Herzing 1997).

The focal population of Atlantic spotted dolphins for this study has been under long-term observation since 1985. Given the amount of observational data collected over recent decades, the study population provides a unique opportunity for the genetic investigation of the mating system and paternity of a small delphinid species. Presented here is the combination of over 20 years of photo-identification data and genetic information for members of a free-ranging population of Atlantic spotted dolphins. The combined data was used to address our first goal, to assign paternity to calves in the population based on known mother–offspring pairs and candidate fathers. Our second goal was to use the resulting paternity assignments to determine whether male reproductive success was random or related to specific male characteristics such as age class or social cluster. Our final goal was to achieve reliable results for this project utilizing a completely noninvasive protocol for genetic sampling and observation.

Materials and methods

Study site and population

The study site is located north of Grand Bahama Island on Little Bahama Bank. It is approximately 480 km^2 and extends 60 km north to south and 8 km east to west. The area is a shallow sand bank (6–16 m depth) with patchy areas of reef and grass. The bank is unprotected and the western edge steeply drops to a depth of >500 m into the Gulf Stream.

The field season consisted of approximately 80–100 field-days every year from May through September utilizing a 21 m power catamaran as the research platform. Observations occurred from 0700 to 2000 during days in the study area, with shorter observation times on days of travel to and from the study area or during times of severe weather. Identification of individual spotted dolphins was accomplished through underwater observation of physical characteristics such as nicks in the dorsal fin, pectoral fins, or flukes; cuts, rake marks; scars; and overall spotting and coloration patterns. The underwater identifications were confirmed by researchers familiar with the resident population through underwater photographs taken of the individuals. Individuals in the population that have been sighted repeatedly were assigned a unique four-letter designation for identification pur-

poses. Mother and calf pairs were assigned through underwater observation of close association, lactation, and nursing.

Since 1985, over 200 individuals have been identified in the study area. Each year, the population consists of an estimated 90–100 individuals. Previous social assessment by Welsh (2007) subdivided the study population into three main social clusters (North, Central, South) based on coefficients of association (COAs) estimated by the half-weight index, principle coordinates analyses (PCO), and latitudinal geographic ranging data gathered from 2002 to 2006. In general, the animals in the North cluster range only in the northern latitudes, while the Central cluster geographically ranges throughout the central and northern latitudes. The South cluster has the largest geographic range and the South animals have been sighted throughout the southern, central, and northern latitudes. Although their geographic ranges overlap, the animals in each of the social clusters associated more with each other than with individuals from other clusters. Based on COAs, PCO, and ranging data, there was a group of individuals (n = 10) that did not fit well with any cluster previously defined by Welsh (2007). Geographically, the 10 individuals ranged throughout all three areas, similar to the South cluster. These unclassified animals did associate with individuals of the Central and South clusters, but they most frequently associated with each other. The unclassified animals were categorized together as a fourth group for the purpose of this study and are referred to as the Roaming cluster. Although the social cluster assignments from Welsh (2007) representing the long-term social trends of individuals in the population were predominantly utilized in the present study, there were samples collected from some individuals not included in the previous study. For this study, those individuals were assigned a social cluster based on the number of times that they were sighted with known-cluster individuals. For example, if an individual was most often sighted with North individuals, the animal was included in the North cluster for this study.

DNA extraction and amplification

This study utilized a noninvasive methodology for the collection of DNA from underwater fecal plumes. The long-term behavioral observations of the study population depend heavily on the fact that humans enter the water with the animals to observe and record behavior. The animals are tolerant of human presence in the water and often approach snorkelers within close range. Given the importance of the underwater observations, it was necessary to consider a noninvasive source of genetic sampling to avoid any change in subject tolerance of human presence in the water.

Fecal sample collection, storage, and extraction followed Green et al. (2007). Polymerase chain reaction (PCR) was used to amplify a fragment of the variable 5′ end of the control region of the mitochondrial genome using primers L15824 and H16265 (Rosel and Block 1996; Rosel et al. 1999), following previously reported parameters (Green et al. 2007). All resulting sequences were searched in GenBank to ensure amplification of the target sequence from the DNA.

In addition to mitochondrial sequences, 10 polymorphic microsatellite loci were amplified: *EV37*, *EV01* (Valsecchi and Amos 1996); *D08* (Shinohara et al. 1997); *Ttr04*, *Ttr11*, *Ttr19*, *Ttr34*, *Ttr48* (Rosel et al. 2005); *Ttru* AAT_{44} (Caldwell et al. 2002); *KWM12* (Hoelzel et al. 1998). All loci were optimized for use with fecal material using the PCR Optimizer™ Kit (Invitrogen, Carlsbad, California, USA). Parameters for loci *EV37*, *D08*, and *Ttr48* are previously reported (Green et al. 2007). Reactions were carried out in 25.0 μL volumes consisting of 0.06 mol/L Tris-HCl (pH 8.5), 0.015 mol/L $(NH_4)_2SO_4$, 0.2 mmol/L dNTPs, 0.2 μmol/L primers, 0.75 U (1 U ≈ 16.67 nkat) *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, Philadelphia, USA), and 1.0 μL 1× template DNA except for locus *Ttr34*, which required 0.4 μmol/L primers. The concentration of $MgCl_2$ varied for each locus (1.5–3.5 mmol/L). Amplification parameters for 7 of the 10 loci are shown in Table 1. Parameters for *EV37*, *D08*, and *Ttr48* are previously described by Green et al. (2007).

All microsatellite fragments were initially visualized on a 6% polyacrylamide gel stained with ethidium bromide (EtBr) prior to sizing. DNA fragments were sized on an ABI Prism 310 genetic analyzer using GENESCAN ANALYSIS® version 3.1 and GENOTYPER® version 2.1 (Applied Biosystems, Foster City, California, USA). The data set was checked for duplicate samples with the EXCEL MICROSATELLITE TOOLKIT (available from http://animalgenomics.ucd.ie/sdepark/ms-toolkit/index.php; accessed 13 December 2007).

Genotyping error

Genotyping error has been defined as the observed differences in two or more genotypes obtained independently from the same sample (Bonin et al. 2004). Such error is common especially in studies utilizing noninvasive tissue types such as feces, hair, and feathers (Taberlet and Luikart 1999; Taberlet et al. 1999; Bonin et al. 2004; McKelvey and Schwartz 2004). Genotyping error is problematic because it can result in errantly typing individuals as homozygous when they are in fact heterozygous (Taberlet and Luikart 1999). Errors can arise from samples of very dilute DNA (Taberlet et al. 1996), degraded DNA, preferential amplification of one allele (Taberlet and Luikart 1999), false alleles from PCR artifacts (Taberlet and Luikart 1999), or sample contamination (Rodríguez et al. 2001).

Precautions were taken throughout the project to reduce genotyping error. The computer program GENOTYPER® version 2.1 (Applied Biosystems, Foster City, California, USA) was used to assign allele sizes. Once the program generated an allele size, all of the chromatograms were checked by eye to verify the data. If a sample contained low quantity or excessively degraded DNA, multiple amplifications were completed based on the multiple tubes approach (Taberlet et al. 1996; Miquel et al. 2006). A consensus genotype from all amplifications was reported and used in subsequent analyses.

To estimate the level of genotyping error in the data set, a blind study was conducted. An individual without prior knowledge of sample genotypes randomly selected 12 samples (approximately 13% of the total sample set) and relabeled those samples according to a new, unique labeling system. The 12 samples were provided to the first author for use in the blind study. The blind-study samples followed the same protocol that had been used for sample processing, in

Table 1. Polymerase chain reaction (PCR) thermal cycler profiles run for amplification of microsatellite loci in fecal material of Atlantic spotted dolphins (*Stenella frontalis*).

Locus	D_{temp}	D_{time}	Cycles	Temp	Time	Ann_{temp}	Ann_{time}	72 °C	Ext. 72 °C
EV01	90	2 min	10	93	1 min	50.4	1 min	50 s	
			25	90	45 s	53.4	1 min	1 min	5 min
Ttr04	94	30 s	40	94	20 s	60	20 s	40 s	10 min
Ttr11	94	30 s	40	94	20 s	60	20 s	40 s	10 min
Ttr19	94	30 s	40	94	20 s	60	20 s	40 s	10 min
Ttr34	94	30 s	40	94	30 s	58	30 s	30 s	10 min
Ttru AAT_{44}	92	1 min	35	92	15 s	56	15 s	30 s	10 min
KWM12	95	3 min	15	94	30 s	69[a]	30 s	30 s	
			20	92	30 s	54	30 s	30 s	2 min

Note: All temperatures reported in degree Celsius (°C); minutes (min); seconds (s); initial denature temperature (D_{temp}), initial denature time (D_{time}), annealing temperature (Ann_{temp}), annealing time (Ann_{time}), and final extension time (Ext. 72 °C).

[a]Annealing temperature decreased by 1 °C each cycle.

that if the amplification was successful, the allele sizes were scored. If the amplification was questionable for any reason, the sample was re-amplified until a consensus was reached. Questionable samples were re-tested if allele amplitude was close to the baseline or if differences in peak sizes were unclear. Once the allele sizes for all blind-study samples at all 10 loci were determined, they were compared back to the full data set to identify the samples that had been re-tested in the blind study.

To estimate the initial error rate in the data set, the blind-study data were analyzed in a second manner. For each individual, only the initial attempt at PCR amplification and sizing was compared with the consensus (Bonin et al. 2004). For each mismatch in allele size, a score of one was assigned. If there was not a mismatch, a score of zero was assigned. The total mismatch score was divided by the total number of allele comparisons and reported as the error rate.

Error rate was also estimated from genotypes of mothers and calves. It is expected that offspring will share at least one allele at each locus. Allele sizes for mothers and calves were compared across all loci. The number of mismatches was recorded and divided by the total number of allele comparisons. Any mother and calf pairs with true nonfilial mismatches were excluded from the analyses. Finally, the remaining data set was analyzed for the presence of null alleles, large allele drop-out, and stutter bands using MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004).

Statistical analyses

Tests for deviation from Hardy–Weinberg equilibrium (overall deviation, heterozygote deficiency, heterozygote excess) and linkage disequilibrium were carried out using Fisher's exact tests and the Markov chain method (10 000 dememorization steps, 1 000 batches, and 10 000 iterations per batch) using GENEPOP version 3.4 (Raymond and Rousset 1995) and Bonferroni-corrected for multiple comparisons. Input files for GENEPOP were constructed using CONVERT version 1.31 (Glaubitz 2004).

Allele frequencies, number of alleles per locus, and estimates of null allele frequency were calculated by CERVUS version 3.0 (Marshall et al. 1998; Kalinowski et al. 2007), which used an iterative algorithm based on the observed and expected frequencies of the genotypes in the data set to determine null allele frequency estimates (Summers and Amos 1997). Levels of gene diversity were estimated as expected (H_E) and observed (H_O) heterozygosity in CERVUS version 3.0 (Marshall et al. 1998; Kalinowski et al. 2007). The polymorphic information content (PIC) was used as a measure of informativeness for each genetic marker (Hearne et al. 1992) and was calculated using CERVUS version 3.0.

Paternity analyses

Paternity assignment was carried out using CERVUS version 3.0 (Marshall et al. 1998; Kalinowski et al. 2007). The program evaluated hypotheses given a set of data and determined the likelihood of one hypothesis relative to another. The two hypotheses considered were (1) H_1, where the alleged father is the true father, and (2) H_0, where the alleged father is an unrelated random male from the population.

The likelihood score of each hypothesis was compared and used to calculate the likelihood ratio. The likelihood ratios were reported as LOD scores (the logarithm of the likelihood scores). The LOD scores of the two most likely males were compared and the difference was reported as Δ. Paternity was assigned to a male if the likelihood ratio was large relative to the likelihood ratios of alternate males based on critical values determined through simulation runs.

A total of 100 000 iterations were run in the simulation of parentage analyses to estimate the critical values of the LOD statistics and Δ. The simulations used allele frequencies to randomly generate genotypes of a mother and father, and then generated genotypes for offspring through Mendelian sampling. The LOD scores of the true parent and unrelated candidate parents were calculated and CERVUS identified the most likely parent (which may or may not be the true parent). Since the program could check to see if it had assigned the true parent, the simulation determined the critical value of Δ needed to assign paternity when the true parent is not known. These critical values were then applied to the actual parentage analyses.

Input parameters for CERVUS included the proportion of loci typed, genotyping error rate, number of candidate males in the population that could have sired the offspring, and the proportion of candidate males that have been sampled. The proportion of loci typed was calculated from the microsatellite data and based on the results of the genotype error tests previously described, and genotyping error was set to 1% for paternity analyses. The number of candidate males and the

proportion sampled were estimated through field data. The most conservative approach was to estimate the number of males in the population regardless of age that could have had physical access to the sampled females. Our approach utilized sighting data in the field during the years when males were most abundant (i.e., years prior to 2004). The sex ratio in the study population was close to parity (Herzing 1997). During each year in which genetic samples were collected (2000–2007), the number of individuals sighted averaged 93, indicating approximately 45 males in the population each year. However, there was a 36% decrease in the number of individuals sighted the following field season, potentially owing to the number of hurricanes that tracked through the study site in 2004 or other possible factors (Elliser 2010; Elliser and Herzing 2011). Therefore, the mean sighting data prior to 2005 was used as a more conservative overestimate of population size. Prior to 2005, the mean number of individuals sighted per year was 108, indicating approximately 54 candidate males in the population, regardless of age or sexual maturity. Although rates of discovery have previously indicated that the study population is closed (Welsh 2007), the full extent of movement of both males and females in and out of the population is currently unknown. Therefore, paternity was also assessed with 100 candidate males in the population to encompass extreme levels of individual movement in and out of the study population. The proportion of candidate males sampled was tested at three different levels for 54 candidate males, based on the estimated age class of each male, and at a single level for 100 candidate males. Sixty-seven percent of 54 males represented the proportion of males sampled regardless of age (36 total males sampled out of 54 candidate males). Forty-six percent considered only mottled and fused males as potential sires, and 33% considered only fused males. When testing with 100 candidate males, the proportion of sampled males was determined regardless of age class (36 total males sampled from 100 candidates).

CERVUS version 3.0 was flexible with the data in that it took into account the number of candidate males and resolved paternity at a predetermined level of confidence even if some of the candidate males have not been sampled. The program does not require that all individuals be typed at every locus and takes genotyping error into account. In addition, CERVUS assumes negligible levels of inbreeding in the population. Therefore, the inbreeding coefficient F_{IS} (Weir and Cockerham 1984) was calculated using FSTAT version 2.9.3 (Goudet 1995).

In studies of pairwise relatedness, the potential for errors in relationship classification must be carefully addressed and understood. In genetic paternity analysis, there are two types of error that can occur when classifying dyads: a dyad can be falsely labeled as a father–offspring pair (type I error), or a dyad will not be labeled as a father–calf pair when the relationship is in fact true (type II error). Choosing a high confidence level (95%) can decrease type I error but may increase type II error to an unacceptable level. Conversely, choosing an 80% confidence level may actually increase the type I error but decrease type II error (Cerchio et al. 2005). In the present study, paternities assigned at the 80% confidence level were reported and used to determine the patterns in male reproductive success; however, data were carefully checked and no mismatches in allele size were allowed for assigned father–calf pairs.

Age determination

The ages of the mother and assigned father at the time of the conception of the calf were estimated through a combination of observational data and generalized age-class ranges. Since Atlantic spotted dolphins gain spots as they age, age estimates can be made based on the speckling and coloration patterns of individual dolphins. In this study, age ranges of individuals were based on their identified age class at the time that they were first observed in the field. For instance, if an individual was first observed in 1986 in the fused age class, their age range in 2007 was determined to be at least 37+ years because the individual was at least 16 years in 1986, plus an additional 21 years to 2007. If the individual was first observed in the year that they were born, an actual age was reported. In addition, an age class was assigned to each individual based on their spot morphology every year that they were observed in the field. By combining all available information, a reliable estimate of age was reported for each individual. Once paternities were assigned, the data were checked to determine if the assigned sire was of a reasonable age to be considered the father of the calf in question. The male was rejected as the father if he was not old enough to have sired offspring in the estimated year of conception of the calf.

Father or closely related individual?

There are two categories of kinship analysis: (1) relatedness estimators and (2) assignment of dyads to relationship categories (Blouin 2003). Both types of analysis are based on the coefficient of relatedness (r), defined as the expected proportion of alleles that two individuals share identical by descent (IBD) (Roughgarden 1996). Polymorphic genetic markers allow for the estimation of relatedness between individuals and there are a number of estimators available (Van De Casteele et al. 2001; Blouin 2003; Csilléry et al. 2006). However, no genetic estimator of relatedness currently performs consistently better than others in identifying pairwise relatedness of dyads (Van Horn et al. 2008).

In free-ranging populations where pedigree information is unknown, paternity assessment can be complicated by the presence of close relatives in the data set. Based on allele inheritance patterns, certain relationship categories will have the same expected relatedness values. For instance, offspring are expected to share 50% of alleles IBD with their parent ($r = 0.50$). However, full siblings are also expected to share 50% of alleles IBD ($r = 0.50$). Half-siblings and avuncular pairs (any combination of an aunt or uncle with a niece or nephew) are expected to share 25% of alleles IBD ($r = 0.25$), and unrelated individuals are not expected to share any alleles IBD ($r = 0$) (Blouin 2003).

In the study population, it is possible that an assigned father might actually fall into another relationship category with the calf in question. Therefore, other possible relationships that must be considered are (*i*) the assigned father is the true father; (*ii*) the assigned father is a full sibling of the calf; and (*iii*) the assigned father is either a maternal or paternal half-sibling of the calf. On Little Bahama Bank, fe-

males exhibit promiscuous mating patterns (Green 2008), making the possibility of full siblings in the population unlikely. Maternal relationships are well documented and a relatively high proportion of maternal half-siblings are already known. Conversely, no information regarding paternal pedigrees exists and it is possible that the assigned father is actually a paternal half-sibling to the calf. To assess the likelihood of the father–offspring relationship versus a half-sibling relationship, two hypotheses were tested: (1) H_1, where the relationship between the assigned father and calf is true, and (2) H_{HS}, where the two individuals are half-siblings.

The assigned father–offspring data set was tested for both maternal and paternal half-sibling relationships using KINSHIP version 1.3.1 (Goodnight and Queller 1999). The analyses follow the principle that each individual inherits an allele from its mother and father, X_M/X_P and Y_M/Y_P. The allele frequencies in the population were defined as P_{XM}, P_{YM}, P_{XP}, and P_{YP}. The program assigns R values, which are estimates of the coefficient of relatedness (r). Two R values, R_M and R_P represent the probability that the maternal and paternal alleles are IBD. Values of $R_M = 0$ and $R_P = 1$ represent a father–offspring relationship in the primary hypothesis, while $R_M = 0$ and $R_P = 0.5$ represent paternal half-siblings in the null hypothesis. Depending on the pattern of allele matching and mismatching among the four alleles, one of four equations was used to calculate a likelihood value (Goodnight and Queller 1999). The program then compares the likelihood of the primary hypothesis to that of the null and reports the log of the likelihood ratio ($LR_{H1/HS}$).

A total of 10 000 simulations were run to determine the critical likelihood score necessary for statistical confidence. The simulations randomly selected a genotype from the data set, individual X. Individual Y was selected using the values of R_M and R_P that define the relationship of the pair. For Y's first allele, the program either copies X's allele (using probability R_M) or draws a random allele. The second allele is chosen in the same manner, using R_P. The likelihood ratio value that excludes 95% of the null-related pairs corresponds to the $P = 0.05$ significance level. The program also determined the corresponding likelihood ratio values for $P = 0.01$ and $P = 0.001$.

All assigned father–offspring pairs were tested in KINSHIP with the primary hypothesis being that the dyad is a father–offspring pair versus an unrelated pair. Second, assigned father–offspring pairs were tested to see if they were more likely to be maternal half-siblings. Third, all assigned father–offspring dyads were tested to determine if a paternal half-sibling relationship had a higher likelihood. Fourth, given multiple paternity assignments to a single male, the potential paternal half-siblings were compared to determine if the likelihood of a paternal half-sibling relationship was greater than a nonrelative relationship.

Although estimates of relatedness can provide a great deal of information, some inherent disadvantages exist among currently available methods for relatedness estimators (Van Horn et al. 2008). Csilléry et al. (2006) reported consistently high misclassification rates when genetic estimates of relatedness were used. To estimate the accuracy of the classifications made in KINSHIP, 21 known maternal half-sibling dyads (assigned based on observational data and confirmed by mitochondrial haplotype and allele sharing) were tested both with and without maternal information provided to the program to determine if a maternal half-sibling relationship was more likely than no relationship. Additionally, 25 individuals in the data set were expected to have at least one maternal half-sibling based on field observations. The assignments made by KINSHIP were examined and the number of correct maternal half-sibling assignments (based on observational data) and the number of incorrect assignments for each individual were calculated and the mean number of incorrect assignments determined across all 25 individuals.

Results

Sample collection

For this study, samples from 96 individual Atlantic spotted dolphins were collected. A total of six samples were excluded from the study because of poor-quality or low-quantity extractions. Two duplicate genotypes were found in the data set. In both cases, the genotypes were a 100% match and the mitochondrial haplotype also matched and likely represented re-sampling events. One duplicate in each case was excluded from further analyses. The remaining 88 samples consisted of 52 females and 36 males. All samples represented animals that had been individually identified through the photo-identification program. A total of 29 mother–calf pairs were sampled. There were five sets of the mother and a single offspring, seven sets of the mother and two of her offspring, and three sets of the mother and three or more of her offspring.

Of the 51 samples collected from the Central cluster, there were 24 males and 27 females. A total of 15 animals (3 males, 12 females) were sampled from the North cluster and 13 individuals (1 male, 12 females) were sampled from the South cluster. In addition, a total of nine individuals were sampled from the Roaming cluster (8 males, 1 female). Of the 36 total males sampled, the majority were from the Central social cluster ($n = 24$).

Of the 36 males sampled, mean proportion of each age class represented was determined across sampling years (2000–2007). The total number of sightings for the 36 males ranged from 20 to 33 (mean = 28.6). On average, 12% of the sampled males were in the twotone age class, 25% were speckled, 16% were mottled, and 47% were fused.

DNA amplification

A 402 bp fragment of the mitochondrial control region was amplified for all individuals in the data set. A total of seven haplotypes were found. The sequences were searched in GenBank and found to most closely match known haplotypes from tissues of Atlantic spotted dolphins in all cases. The haplotypes for all mother–calf pairs matched and were used as confirmation of mother–calf assignments made based on observational data in the field.

Genotypes were determined across all 10 loci for most individuals ($n = 76$). Genotypes could not be determined for two individuals at locus *KWM12*, two at *EV37*, four at *Ttr19*, and four at *EV01*, giving an overall genotyping success rate of 868 successfully typed loci over 880 attempts (98.7% success). Individuals that could not be typed at a

Table 2. Number of alleles and allele size range for 10 microsatellite loci used to construct multilocus genotypes for Atlantic spotted dolphins (*Stenella frontalis*).

Locus	No. of alleles	Allele range
EV37	4	198–206
D08	3	97–103
EV01	4	134–140
Ttr04	5	106–114
Ttr11	4	201–213
Ttr19	5	184–194
Ttr34	6	177–185
Ttr48	6	125–133
Ttru AAT$_{44}$	2	83–89
KWM12	4	153–175
Mean	4.3	

particular locus were labeled as missing data at that locus and included in the analyses.

Genotyping error results

Among all 12 samples included in the blind study, the replicates resulted in allele typing that matched with 100% accuracy to the previously assigned genotypes. When the initial attempts at amplification in the blind study were scored and compared according to Bonin et al. (2004), four differences out of 240 comparisons were found, giving an error rate of 1.67%. Of the four differences, three were cases of false alleles and the fourth was error owing to allelic drop-out. An estimated error rate of 1.67% is probably higher than the actual rate present in the data because it does not account for the multiple comparisons made to determine consensus genotypes, which resulted in the final assessment of error as 0% in the blind study.

Genotyping error was also estimated from mother–calf comparisons assuming Mendelian inheritance patterns. The data set originally included 30 mother and calf comparisons. When the pairs were compared for allelic mismatches, one of the comparisons contained a true nonfilial mismatch, confirmed through multiple amplifications of both mother and calf at the locus in question. This mother–calf pair was excluded from further analyses. Excluding 1 pair out of 30 pairs was a rate of approximately 3.3% exclusion. Prior to removing the nonfilial mismatch pair, all 30 mother–calf pairs were compared across all loci. There were four differences out of 293 comparisons, resulting in 1.37% error rate. After the nonfilial mismatch pair was removed, there were three differences out of 284 comparisons, which gave a final error rate of 1.1%. Based on the results of both the blind study and the comparison of mother–calf pairs, the best estimate of genotyping error in the data was 1% and an overall estimate of 1% was used for paternity analyses in CERVUS. Further checks for genotyping error using MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004) resulted in no evidence of null alleles, large allele drop-out, or error detected owing to stutter bands.

Statistical analyses

All 10 loci conformed to Hardy–Weinberg expectations and no significant linkage between loci was detected after Bonferroni correction. The number of alleles per locus ranged from 2 to 6, with a mean of 4.3 (Table 2). Expected heterozygosity (H_E) ranged from 0.351 to 0.727, with a mean of 0.541 (Table 3). Observed heterozygosity (H_O) ranged from 0.369 to 0.698 (mean = 0.519) (Table 3). The PIC ranged from 0.321 to 0.673, with a mean of 0.485 (Table 3). Overall population F_{IS} was <0.05, indicating a low incidence of inbreeding in the population.

Table 3. Observed heterozygosity (H_O), expected heterozygosity (H_E), polymorphic information content (PIC), and estimates of null allele frequencies for 10 microsatellite loci of Atlantic spotted dolphins (*Stenella frontalis*) (F_{IS} = 0.041).

Locus	H_O	H_E	PIC	Null allele frequency
EV37	0.512	0.595	0.541	0.0805
D08	0.386	0.484	0.398	0.1200
EV01	0.369	0.401	0.364	0.0168
Ttr04	0.580	0.543	0.488	–0.0401
Ttr11	0.557	0.589	0.510	0.0227
Ttr19	0.667	0.687	0.638	0.0113
Ttr34	0.375	0.351	0.338	–0.0457
Ttr48	0.602	0.627	0.580	0.0177
Ttru AAT$_{44}$	0.443	0.404	0.321	–0.0490
KWM12	0.698	0.727	0.673	0.0148
Mean	0.519	0.541	0.485	

Paternity analyses

A total of 29 offspring were candidates for paternity testing. Initially CERVUS assigned 12 paternities at the level of 54 potential males with 0.46 sampled and 13 paternities at the level of 54 males and 0.67 sampled. Paternity assignments were rejected based on age for 2 of the 13 father–offspring pairs, Fine–Brus and Scqe–Snow. Calf Brus was born in 1990, whereas the estimated year of birth of the assigned father (Fine) was 1991–1995, making the father younger than the calf. In the second case, Snow was born in 1987 and the year of birth of the candidate father was estimated to be from 1981 to 1985. Based on this estimate, Scqe would have been from 1 to 5 years at the time that Snow was conceived and it is unlikely that such a young male would successfully reproduce.

One additional father–calf relationship (Nave–Lava) was rejected because it was more likely that they were maternal half-siblings ($LR_{H1/HS}$ = 0.261, critical value = 0.648). For this pair, CERVUS originally assigned paternity with relaxed confidence when the data was tested as 54 candidate males and 0.67 proportion sampled. At the more likely level of 46% of 54 potential males sampled, Nave was reported as the most likely candidate male, but paternity could not be assigned because the Δ value was smaller than the critical value (Δ = 1.72, critical Δ = 2.47).

After adjusting the paternity assignments for age and maternal sibling relationship, 10 paternity assignments remained for the 29 offspring tested (34.5%) (Table 4). As expected, the success rate for paternity assignment varied depending on the number of potential candidate fathers in the population and the proportion of those males that had

Table 4. Paternity assignment of Atlantic spotted dolphins (*Stenella frontalis*) based on likelihood ratios (LOD score) and Δ values.

					Proportion of candidate males sampled			
Offspring	Mother	Candidate father	LOD	Δ	0.67[a]	0.46[a]	0.33[a]	0.36[b]
Free	Flyi	Sick	8.34	8.13	*	*	*	*
Pica	Pain	Bigg	8.54	7.91	*	*	*	*
Nept	Nass	Flay	6.76	6.76	*	*	*	*
Hava	Hedl	Surg	6.30	6.30	*	*	*	*
Ditt	Dos	Rome	5.77	5.77	*	*	*	+
Tyle	Trim	Rome	5.62	4.90	*	+	+	+
Mali	Mugs	Surg	4.97	3.61	+	+	+	–
Arep	Appl	Nave	2.98	2.98	+	+	–	–
Vega	Venu	Hors	2.83	2.83	+	+	–	–
Lhas	Lgsh	Bigg	5.15	2.86	+	+	–	–

Note: *, strict confidence (95%); +, relaxed confidence (80%); –, most likely father but paternity not assigned. Shaded column indicates most likely scenario of potential males in population based on observational field data.

[a]There were 54 candidate males in the population.

[b]There were 100 candidate males in the population.

Table 5. Estimated ages and morphological age classes of mothers and candidate fathers of Atlantic spotted dolphins (*Stenella frontalis*) at time of offspring conception.

Offspring	$Year_c$	Mother	Age_c	Age class	Father	Age_c	Age class	Difference
Nept	1998	Nass	9	S	Flay	20–26	F	11–17
Pica	1998	Pain	17–21	M	Bigg	28+	F	7–11+
Tyle	1998	Trim	14–18	M	Rome	29+	F	11–15+
Lhas	2000	Lgsh	18–22	M	Bigg	30+	F	8–12+
Vega	2002	Venu	14–18	M	Hors	19–23	F	1–9+
Hava	1990	Hedl	20+	F	Surg	20+	F	0+
Ditt	1995	Dos	18–24	F	Rome	26+	F	2–8+
Mali	1999	Mugs	17–21	F	Surg	29+	F	8–12+
Free	2001	Flyi	20–24	F	Sick	26+	F	2–6+
Arep	2004	Appl	25+	F	Nave	18	F	(–7)
Mean (range)			18.7+ (17.2 to 20.2+)			25+ (23.5 to 25.5+)		7.7+ (0 to 9.5+)

Note: Estimated year of conception of offspring ($year_c$), estimated age of individual at time of conception (age_c). Age classes indicate speckled (S), mottled (M), and fused (F).

been sampled. A total of five paternities were assigned with strict confidence (95%) and five with relaxed confidence (80%) when the number of candidate males was low (n = 54) and the proportion of sampled males was 46% (Table 4). The number of paternities assigned decreased when 100 candidate males were assumed and only 36% were sampled. At this level, six paternities were assigned (four with strict confidence, two with relaxed).

In all cases, the assigned father shared an allele at each locus with the offspring. In 9 of 10 cases, all 10 loci were typed and compared across the father and offspring. In a single pair, only 9 loci were typed for the calf, therefore only those loci were compared.

Age at conception

At the 80% confidence level, a total of 10 paternities were assigned to seven males (Table 4). Half of the females (n = 5) were in the oldest age class at conception, four females were in the mottled age class, and a single female was in the late-speckled phase. The youngest female was 9 years of age and the oldest female was estimated to be from 25+ years of age (mean = 18.7+ years) (Table 5).

Based on the estimated age of the mother and assigned father at conception, the male was almost always older than the female (80%) (Table 5). In a single pair, the male was younger than the female, although the male was still in the oldest age-class category (fused). All seven males that were assigned paternities were in the fused age class (16+ years) at the time of the conception of the calf. The males ranged in age from 18 to 30+ years (mean = 25+ years), with 18 years being the estimated age of the single male (Nave) that was younger than the female that he conceived a calf with. Of the males that were older than their female counterpart, the males were, on average, at least 7.7 years older than the females (Table 5).

Half-sibling likelihood

In all cases, the assigned father–calf relationships were more likely than being unrelated individuals according to KINSHIP ($P < 0.001$ to 0.05). The 11 father–calf assignments

Table 6. Likelihood of father–offspring relationship versus no relationship and paternal half-sibling relationship among Atlantic spotted dolphins (*Stenella frontalis*).

		Father vs. unrelated		Father vs. paternal half-sibling	
Offspring	Candidate father	$LR_{HP/HO}$	Critical value	$LR_{HP/HO}$	Critical value
Free	Sick	5.280***	2.0209	1.631***	1.1653
Pica	Bigg	3.763***	2.0209	1.262***	1.1653
Nept	Flay	3.079***	2.0209	1.072**	0.902
Tyle	Rome	2.494***	2.0209	0.985**	0.902
Ditt	Rome	2.695***	2.0209	0.921*	0.902
Lhas	Bigg	2.056***	2.0209	0.654*	0.646
Hava	Surg	2.295***	2.0209	0.646*	0.646
Vega	Hors	1.714**	1.2855	0.549	
Mali	Surg	1.906**	1.2855	0.512	
Arep	Nave	1.036*	0.4994	0.400	

Note: Likelihood ratio of hypothesis indicating that the father–offspring relationship is true versus the pair being two unrelated individuals ($LR_{HP/HO}$) and likelihood ratio of hypothesis indicating that the father–offspring relationship is true versus a paternal half-sibling relationship ($LR_{HP/HO}$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 7. Social cluster assignments of mother–calf pairs and assigned fathers of Atlantic spotted dolphins (*Stenella frontalis*).

Offspring	Mother	Range[a]	Candidate father	Range
Pica	Pain	North	Bigg	Central
Lhas	Lgsh	Central	Bigg	Central
Ditt	Dos	Central	Rome	Central
Tyle	Trim	North	Rome	Central
Hava	Hedl	Central	Surg	Central
Mali	Mugs	Central	Surg	Central
Arep	Appl	Central	Nave	Central
Nept	Nass	Central	Flay	Roaming
Free	Flyi	South	Sick	Roaming
Vega	Venu	South	Hors	Roaming

[a]Range for mother represents the social cluster for both mother and calf.

were tested to determine if the father–calf assignment was true or if a maternal half-sibling relationship was more likely, and 1 of the 11 dyads was more likely to be maternal half-siblings (Nave–Lava: $LR_{HP/HO} = 0.261$, critical value = 0.648). Seven of the assigned father–offspring pairs were more likely to be true relationships rather than paternal half-siblings ($P < 0.05$) (Table 6). Given the microsatellite data set, three assigned father–offspring pairs were more likely to be paternal half-siblings ($LR_{HP/HO}$: range 0.4–0.549, critical value = 0.646) when testing the two hypotheses in KINSHIP. Although there are conflicting results from KINSHIP, these three paternity assignments remain assigned as father–offspring pairs. The maternal half-sibling relationship was accepted and the earlier paternity assignment was rejected with justification for these four decisions presented in the Discussion.

Because three males were each assigned two paternities, the shared offspring would be paternal half-siblings if the father–offspring assignments were true. Three dyads were tested to determine if the two individuals were more likely to be paternal half-siblings or unrelated. One pair (Ditt–Tyle) was significantly more likely to be paternal half-siblings ($\Delta = 0.82$, critical $\Delta = 0.53$, at $P < 0.05$). The two remaining pairs (Pica–Lhas, Hava–Mali) were more likely to be unrelated in comparison to paternal half-siblings ($\Delta = 0.445$, $\Delta = 0.175$, respectively, at critical $\Delta = 0.53$ for $P < 0.05$).

Finally, 21 known maternal half-sibling pairs (based on observational data) were tested to determine if KINSHIP would correctly identify the dyads. Out of 21 pairs, 18 were correctly identified when maternal assignment information was provided (86%), but only 12 were identified when no maternal information was provided (56%). A total of 25 individuals were expected to have one or more maternal half-siblings assigned. On average, KINSHIP assigned an additional 5.88 maternal half-siblings per individual (range 2–10).

Geographic range and social cluster

Five individuals (5.7%) included in the present study were not in the study by Welsh (2007) and were assigned a social cluster based on the number of times that they were sighted with known-cluster individuals. Of the seven males assigned paternity, four were designated to the Central cluster (Table 7). The Central males most often mated with females from the same social cluster ($n = 6$). Three males in the Central cluster were each assigned two paternities. One such male mated with two different females from the same social cluster to produce two offspring. The other two multiple paternity males sired one calf with a female from the same social cluster, and one calf with a female from the Northern cluster. The remaining three males were assigned to the Roaming cluster. In two cases, Roaming males mated with Southern females. One Roaming male was assigned paternity for a Central female's offspring.

Discussion

DNA extracted from underwater fecal plumes was useful in determining paternity in Atlantic spotted dolphins. All derived mitochondrial haplotypes were found to closely match previously published sequences from known tissues of Atlantic spotted dolphins in GenBank. Comparing error rate through both a blind study and Mendelian inheritance patterns provided a more complete picture of overall error rate for the study. Specific precautions and careful testing reduced the amount of genotyping error in the data set. It

should be noted that the error rate could be decreased from 1.67% to 0% through careful examination by eye of each sample. Although this type of checking can be tedious and does not lend itself well to very large data sets, in cases with relatively small sample sets or in cases where relationship assignments depend on microsatellite sizing accuracy, it is well worth the effort to decrease the error rate. When conducted with thorough methods to reduce error, our study supports the evidence that the use of noninvasively collected fecal material from a dolphin species is a reliable method for obtaining quality genetic template DNA (Parsons et al. 1999; Parsons 2001; Green et al. 2007).

Although CERVUS assigned paternity for only a portion of the tested offspring, there are four conclusions that warrant additional discussion. First, reproductive success was skewed towards older males (mean age = 25+ years). Second, males most often mated within their social cluster but were not exclusive. Third, at the 80% confidence level, paternities could only be assigned for 34.5% of offspring. And fourth, this study was similar to others in that some pedigree and demographic information on the population provided a more inclusive picture of relatedness than genetic markers alone.

(*i*) Male age

Male reproductive success was clearly influenced by age. Among the seven males that were assigned paternities, all were in the oldest age class (fused) during the estimated year of conception of the calf. Age is often a significant trait in reproductive success and is usually correlated with body size or possessing larger traits that are important in male–male competition (Coltman et al. 2002). Larger body size or physical characteristics can benefit males of species with high levels of intrasexual competition (Haley et al. 1994) whether they are fighting for direct access to females or fighting to defend indirect resources that are important to females. On Little Bahama Bank, food and protected habitat are not clustered, leaving little opportunity for males to control access to physical resources. The three-dimensional aspect of their ocean habitat limits the ability of males to control access to females. Such factors result in a low environmental potential for polygamy (Emlen and Oring 1977) and forces males to employ different methods to maximize their reproductive fitness.

Several alternate hypotheses can be proposed to explain the bias in age. Females may be actively choosing to mate with older males when they are reproductively receptive; age may correlate with social pairings that increase reproductive success; or age may correlate with increased development of reproductive physiology in males. Observations in the field have revealed that mating and courtship occur within and among all age classes, but females are more often sighted copulating with older males (Herzing 1997). A number of studies exist suggesting that females prefer to mate with older males (Kokko and Lindström 1996), but the reason for the preference is debated. The widely accepted good genes hypothesis proposes females choose older males because age is an indicator of overall viability (Trivers 1972). Female preference for older males explained by the good genes hypothesis depends on age-specific survival rates (Beck and Powell 2000). If juvenile survival rates are high and adult survival rates are low, older males will have higher viability and preference for older males is subsequently expected to evolve in the mating system (Beck and Powell 2000). Conversely, if juvenile survival rate is low compared with adults, then mean viability does not differ among males of different ages and female preference for older males is not likely to evolve in the population (Beck and Powell 2000). Hansen and Price (1995) argued that young to intermediate-aged males have the highest breeding values for fitness. That would make the intermediate-aged males of higher genetic quality than older males, and females should prefer younger males instead of older males. According to a simulation model used by Beck and Powell (2000), in species where males only contribute sperm, female preference based on age is most likely to evolve in a population if that age preference is directed toward younger and intermediate-aged males. On Little Bahama Bank, male Atlantic spotted dolphins do not participate in calf rearing, therefore if there is female age preference of males, according to Beck and Powell's (2000) simulation, we would expect to see at least some paternities assigned to males in the mottled age-class category, not just in the fused category. As no paternities were assigned to mottled males, a better hypothesis to explain the age bias in reproductive success might encompass a physiological or social constraint on younger (mottled) males, rather than the choosiness of females.

Age is often a precursor to achieving a high-ranking position in social species where dominance correlates with reproductive opportunity (Widdig et al. 2004) and age may correlate with social standing in cetacean societies. Social animals form alliances, or long-term associations with one or multiple individuals (Harcourt and de Wall 1992). In Shark Bay, Australia, alliance formation can lead to an increase in reproductive success among male bottlenose dolphins (Krützen et al. 2004). On Little Bahama Bank, male alliance patterns similar to Shark Bay have been observed among Atlantic spotted dolphins. Alliances solidify during the mottled years and the strength of those alliances may increase as the animals advance into the fused age class (COA $\geq$ 0.7) (C. Elliser, personal communication (2009)). Brunnick (2000) tested for the presence of male alliances in the study population from 1990 to 1997 and identified 25 males that were associating in four distinct alliance groups. A total of four of the seven males that sired offspring in the current study were in an alliance from 1990 to 1997. The four males sired offspring from 1990 to 2000 and all four males were sighted multiple times each year from 1998 to 2000 (long-term alliances are unlikely to change in a 2-year period). Such preliminary correlations may indicate that having an alliance and stable associates may be important in gaining reproductive opportunities. Updated analysis of associations in the population will help resolve the social status of males correlated with their reproductive success. However, the hypothesis of having a social alliance as a benefit to reproductive success is feasible.

Physical development of older males may increase reproductive success. An increase in androgens (testosterone and metabolites) during postnatal development is key to defining puberty in mammals (Preslock 1980). Androgens are used as indicators of male reproductive maturity in many mammals,

including cetaceans (Kellar et al. 2009). Rolland et al. (2005) found adult (10–26 years) male North Atlantic right whales had higher levels of androgen hormones compared with juveniles (2–9 years). The mean fecal androgen levels were less than one-half in juveniles and could represent sexual maturation of North Atlantic right whales as early as age 10 (Rolland et al. 2005). Frasier et al. (2007) found in North Atlantic right whale, older males have higher reproductive success than younger males. In fact, most males did not successfully sire a calf until 15 years of age (Frasier et al. 2007), which may be 5 years after males are physically capable of producing sperm (Rolland et al. 2005).

Whereas the age of first reproductive success in male Atlantic spotted dolphins is unknown, Perrin (2001) estimated that male pantropical spotted dolphins reach sexual maturity between 12 and 15 years of age, an estimated 3 years after females reach maturity; on the other hand, Hohn et al. (1985) estimated males, on average, reach sexual maturity at 14.7 years of age. The oldest reported immature pantropical spotted male was estimated to be 16 years old, and the youngest mature male was estimated to be 8 years old (Kasuya et al. 1974; Kasuya 1976). In this study, 18 years was the youngest estimated age of a male at the time of calf conception. There may be varying degrees of testes size and development, meaning that males might be sexually mature and capable of sperm production at an early age, but further years of development will continue to increase testes size (Frasier et al. 2007). In a study of gonad sizes in male common dolphins (*Delphinus delphis* L., 1758), the youngest sexually mature male was 8 years but most were 10 years and older (mean = 16.7 years) (Murphy et al. 2005). Mean blubber testosterone levels of common dolphins were significantly higher in mature males than in pubertal or immature males (Kellar et al. 2009). There was evidence of seasonal variation with higher levels of blubber testosterone in the summer (Kellar et al. 2009). Although some males may attain sexual maturity at an early age, most males may not reach the necessary stage of testicular development until their late teens. Murphy et al. (2005) reported seasonal of variation in testes size of common dolphins, indicating a "rut" or seasonal breeding period. On Little Bahama Bank, older fused males have been observed with enlarged keels or postanal swelling, which appeared similar to enlarged mammary glands of lactating females. Keel swelling may indicate that older Atlantic spotted males enter a state of "rut", which includes swollen testes, and consequently, increased sperm production. However, the level of testosterone in younger males may not be adequate to significantly increase sperm production and may account for the skew in reproductive success.

(*ii*) Social cluster preference

All paternities were assigned to males from either the Central or the Roaming clusters. However, the data should be interpreted cautiously because of the limited sampling from the North and South clusters in this study. Based on previous social analysis, the Central social cluster was the largest cluster (52 total: 27 male, 25 female), whereas the South and North clusters were smaller with 16 and 15 individuals, respectively (South: 13 female, 1 male, 2 unknown; North: 12 female, 3 young juvenile males) (Welsh 2007). Therefore, the fact that males in the Central cluster sired the majority of calves was expected, as the highest proportion of males in the population came from the Central cluster. Roaming males were assigned paternity for three calves. The Roaming males mated with females from either the Southern or the Central clusters, which was also expected because the Roaming males are frequent associates of the Southern and Central clusters, even though they most frequently associate with each other (Welsh 2007). Interestingly, two males assigned multiple paternities sired one calf within their own social cluster and a second calf with a female from outside the cluster. Bigg and Rome each sired a calf with a Northern female. In general, the pattern of mating seems to be that the largest cluster, the Central cluster, is somewhat self-contained. The number of males and females in the cluster is sufficient to support mating within the cluster. On the other hand, the South and North clusters consist primarily of females. To be reproductively successful, these females need to mate with males outside of their social cluster. The Central cluster is the closest group geographically to the North cluster and it is known that Central individuals move throughout both the Central and the North latitudinal ranges (Welsh 2007). Females show behavioral evidence of site fidelity and cluster fidelity (Herzing 1997; Welsh 2007), as well as genetic evidence (Green 2008). Males may be ranging around more than females to increase their access to females, and subsequently, increase their reproductive success by going into the female ranging areas. Future analysis of social clustering across a wider range of years is required to clarify the mating preferences of males as it relates to social clusters. However, the preliminary data suggests there is a general pattern that Central males mate with Central and North females, whereas the Roaming males mate with South and Central females.

(*iii*) Paternity assignment

Paternity could only be assigned for 34.5% of the study population offspring tested. The reason for the relatively low level of paternity assignment could be due to several factors. One reason could be the number of loci or degree of polymorphism of the molecular markers used. By testing additional loci or loci with higher levels of polymorphism, the statistical confidence may be increased enough to assign additional paternities. The assignment rate could also be low if the fathers of the remaining offspring were not sampled. Although it was estimated that there are approximately 54 candidate males in the population, only 36 males total were sampled for the study. The low proportion of males sampled could be attributed to several factors.

First, the fathers that have not been sampled could be dead or absent from the study area. There are no reliable estimates of mortality rate for the population because carcass recovery is exceptionally unlikely. The study site is far from shore and predators or scavengers likely consume bodies long before they can drift to a beach area where humans would see it. Second, there may be sex-based differences in fecal sampling. Females have a consistently higher re-sighting rate than males in the largest cluster (Central) (C. Elliser, personal communication (2009)), which makes the chances of sampling males lower than that of females. The low proportion of sampled males could result from males

moving in and out of the population. Although the population is generally considered closed (Welsh 2007), rates of immigration and emigration are currently unknown. Short-term absences lasting at least 1 year are known to occur and some animals actually exhibited an alternating pattern of presence and absence over several years (Brunnick 2000). One male, Heli, was first seen in the fused age class in 1995. During this year, he was sighted on four different dates. In 1996, he was sighted on a single occasion and then he was not sighted from 1997 to 2005. In 2006, Heli was re-sighted after a 9-year gap in sightings. Heli may have left the regular geographical ranging area of the resident population during those 9 years and later returned. Although this sighting pattern is not common among the study population, it is clear that there are some cases of movement in and out of the group. In Sarasota, Florida, an estimated 15% of calves of bottlenose dolphins were fathered by nonresident males (Duffield and Wells 2002). The nonresident males may have encountered females in the study population either because the males were in a neighboring or transient group or because females left the area to seek out males (Duffield and Wells 2002). Genetic evidence of female-mediated gene flow has been reported for bottlenose dolphins in the Bahamas (Parsons et al. 2006). Therefore, it is possible that individuals of either sex may leave the population for periods of time prior to returning to the group, although movement of females is less likely given the level of female site fidelity on Little Bahama Bank (Green 2008).

(*iv*) Constructing a complete picture of relatedness

Estimates of relatedness between dyads can be obtained through pedigrees, but complete pedigrees are rarely available to researchers working with free-ranging populations. Polymorphic genetic markers such as microsatellites allow for an estimation of relatedness for individuals with unknown ancestry (Van De Casteele et al. 2001). Demographic data, genetic data, and pedigree data combined together gives the best and most logical picture of pairwise relatedness (Van Horn et al. 2008) in our study.

KINSHIP correctly revealed one previously unknown pair of maternal half-siblings. The pair was first weakly assigned as a father–calf relationship, but the likelihood of maternal half-siblings was greater than a father–calf relationship. The male, Nave, was assigned as a maternal half-sibling to Lava. Observational and mtDNA haplotype sharing had previously confirmed that Lill is the mother of Lava; therefore, Lill must also be the mother of Nave if the relatedness classification is true. Once the assignment was made in KINSHIP, the possibility of the relationship was checked using all possible resources. In support of the assignment, Nave shares an allele with Lill at all 10 tested loci and shares the same mtDNA haplotype as his mother. Also, given the long-term photo-identification program of the study population, photographic confirmation was made using natural markings (dorsal fin shape, scars, and overall coloration pattern) on Nave compared with previous "lost" calves of Lill. Consequently, combining multiple data types provides the most reliable picture of relatedness within natural populations.

Although KINSHIP correctly identified the maternal half-sibling dyad, the overall performance of the relatedness estimator followed the pattern reported for other studies where some pedigree information existed. The current methods available for estimating relatedness have been shown to have a tendency to mislabel relationships when there is no prior information of pedigrees or demographic data (Van Horn et al. 2008). Csilléry et al. (2006) reported that dyads of previously known pedigree relationships were often misclassified if the classifications were based solely on genetic estimates and our data set was no different. Specifically, Csilléry et al. (2006) reported consistently high misclassification rates and the tested methodologies were overestimating relationships in the tested populations. Overall, the tendency was for dyads to be classified as closer kin than they really were. In a population of savannah baboons (*Papio cynocephalus* (L., 1766)), the overall success rate of KINSHIP was 82.1%, but it varied widely depending on the relationship being tested. Parent–offspring dyads were wrong 71.8% of the time; full siblings were wrong 97.1% of the time with most incorrectly labeled as half-siblings or unrelated; half-siblings were wrong 58.6% of the time with most labeled as unrelated; but unrelated dyads were only incorrect 3.5% of the time (Van Horn et al. 2008).

Of the 10 paternity assignments, 3 were not significantly supported by comparing the likelihood of the father–calf relationship to the likelihood of a paternal half-sibling relationship. Additional testing of paternal half-sibling relationships indirectly supported the father–calf assignments of Rome–Ditt and Rome–Tyle because KINSHIP assigned Ditt and Tyle as paternal half-siblings. CERVUS assigned paternity to Bigg for both Pica and Lhas. However, KINSHIP did not assign a relationship of paternal half-siblings to Pica and Lhas even though both CERVUS and KINSHIP previously supported the father–offspring relationship. The same result was observed for Hava and Mali with Surg as the assigned father. The individuals were more likely to be unrelated than paternal half-siblings, which does not support the father–calf assignment made by CERVUS. The likelihood of the paternal half-sibling relationship was close to the critical value but not high enough to be significant.

Given the discrepancies between paternity assignments and half-sibling assignments and the known problems with type I and type II error with relatedness estimators, the decision to reject father–offspring relationships in favor of paternal half-siblings should be approached cautiously for many reasons. First, Marshall et al. (1998) addressed the problem of full and half-siblings in the population when testing for paternity and stated that the likelihood method utilized in CERVUS is insensitive to even large numbers of half-siblings of the true father within the candidate pool of males. Also, if the mother is sampled with the offspring, relatives of the offspring in the candidate pool do not affect the number of paternities assigned, but the confidence with which those assignments are made slowly decreases as the number of relatives in the candidate pool increases. Although full siblings tend to have a higher likelihood of paternity than the true father, full siblings are less likely than half-siblings in the Little Bahama Bank population given the promiscuous mating behavior of Atlantic spotted dolphins (Green 2008). Second, there was a positive $LR_{H1/HS}$ for all assigned fathers; however, three were not sufficiently large enough to be considered significant (Table 6).

Even though the $LR_{H1/HS}$ was not large enough to support the primary hypothesis of father–calf relationship, the $LR_{H1/HS}$ in each case was positive and was not close to zero (mininum value = 0.4). Third, if the animals in question were actually half-siblings, mismatches of alleles would be expected at a higher rate than if they were father–calf pairs because $r = 0.5$ for parent–offspring pairs and $r = 0.25$ for half-siblings. In fact, there were no observed mismatches between the father–offspring and paternal half-sibling pairs across all 10 loci. Finally, the critical values determined for each level of significance were determined through simulation runs. According to Goodnight and Queller (1999), the power of the method used in KINSHIP is dependent on the relationships being compared. When testing paternity as the primary hypothesis versus half-sibship as the null hypothesis, five loci are necessary to accept 50% of the pairs related as father and calf ($P < 0.05$). The theoretical framework of the number of loci required was based on simulations with 20 equally frequent alleles per locus. Although 10 loci were used in the current study, the mean polymorphic information content was 0.485. If 10 loci with 20 equally frequent alleles per locus were used, the mean PIC would be 0.948. Given the relatively low PIC values, additional loci would be needed to determine if the three nonsignificant father–offspring pairs would shift towards a likelihood ratio that favors either a father–offspring relationship or a paternal half-sibling relationship. However, obtaining precise estimates of R with low variance may require tens or hundreds of microsatellite loci (Csilléry et al. 2006; Van Horn et al. 2008). The number of loci in the study appears to be high enough because at 46% of 54 males sampled, there were no cases where more than one candidate father was assigned.

Conclusion

The noninvasive technique proved useful in providing genetic material for molecular investigations. The sampling protocol allowed for the collection of genetic material while protecting the integrity of ongoing underwater observational studies. Although the percentage of paternities assigned was somewhat low, it was within the range of other paternity studies and future work will add to the current data available for the study population. Additional samples from candidate males may result in future assignments of paternity. With an increased data set of father–offspring pairs, the patterns of correlations between age and reproductive success could be tested further. Additional markers designed specifically for Atlantic spotted dolphins could provide more informative markers to further assign paternity and differentiate between levels of relatedness. In the meantime, our study has provided new information that will prove useful to a diverse range of cetacean species and aquatic mammals.

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