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Molecular identification of individual North Atlantic right whales (*Eubalaena glacialis*) using free-floating feces

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ABSTRACT

During the 1990s, North Atlantic right whales had significantly decreased reproduction and showed signs of compromised health, prompting the initiation of noninvasive fecal-based studies to investigate potential causal factors. The interpretation of these studies is enhanced when the defecator is identified, as data can then be linked to individual life history information. Fecal samples ($n = 118$) were either collected from single photoidentified whales, associated with several individuals by photoidentification of whales in the vicinity upon sample collection, or were collected when no whales were in the vicinity. Genetic profiles from fecal DNA comprising sex, mitochondrial haplotype, and five microsatellite loci helped assign specific samples to individual right whales based on existing genetic profiles. Profiles were informative in assigning 61 fecal samples to known individuals, 24 of which were collected when no whales were in the vicinity. Whales identified genetically were typically photographed in the same habitat area and on the same day of sample collection ($n = 35/48$). Twelve profiles new to the genetic database were identified, suggesting fecal sampling provides a means to obtain genetic profiles from previously unsampled individuals, which may help

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refine estimates of population size and habitat use patterns if annual fecal sampling continues.

Key words: North Atlantic right whale, *Eubalaena glacialis*, fecal genotyping, individual identification, probability of identity.

The North Atlantic right whale (*Eubalaena glacialis*) is one of the world's most endangered whales, with approximately 350–400 individuals remaining (IWC 2001, Kraus and Rolland 2007); although recent genetic analyses suggest that this may be an underestimate of the actual population size (Frasier *et al.* 2007*a*). This species has been internationally protected since 1935 (IWC 1986), and has been the focus of conservation and recovery efforts for the last three decades (Kraus and Rolland 2007). Human-associated mortality from vessel strikes and entanglements in fishing gear are currently recognized as being the main factors impeding recovery (Kraus 1990, Knowlton and Kraus 2001, Kraus *et al.* 2005, Moore *et al.* 2005, 2007). In addition to these direct anthropogenic threats, several signs of reproductive dysfunction are evident, further limiting population growth (Kraus *et al.* 2001, 2007).

During the 1990s, the North Atlantic right whale exhibited signs of poor health (Pettis *et al.* 2004, Hamilton and Marx 2005, Rolland *et al.* 2007*a*, *b*) and highly variable annual calving rates (Kraus *et al.* 2001, 2007), prompting the initiation a fecal-based research program to assess specific factors that could be influencing health and reproduction (*e.g.,* Rolland *et al.* 2007*b*). To date, the collection and analysis of fecal samples have provided researchers with a wealth of information on the health and reproductive status of individuals that was not previously available, including information on parasite loads and marine biotoxin exposure (Doucette *et al.* 2006, Hughes-Hanks *et al.* 2005, Rolland *et al.* 2007*b*). In addition, studies using fecal reproductive hormone metabolites to determine sex, detect pregnancy, and lactation, and assess age at sexual maturity in males have been validated (Rolland *et al.* 2005, 2007*a*), as have assays for fecal glucocorticoid metabolites as a quantitative measure of physiologic stress (Hunt *et al.* 2006).

Although data obtained from the analyses of fecal samples have been extremely valuable, interpretation of the results is enhanced when samples are associated with known individuals. Right whales are identified using photographs through a combination of callosity patterns on their rostrum, lips, and chin, and distinctive pigmentation, scars, and marks on their bodies (Kraus *et al.* 1986, Hamilton *et al.* 2007). Photographs of individual whales have been collected for three decades by over 200 organizations and individual researchers (Kraus *et al.* 1986, Hamilton *et al.* 2007), and all sightings are currently archived in the *North Atlantic Right Whale Catalog* (Hamilton and Martin 1999, Hamilton 2007). Additionally, genetic analyses have been conducted on skin biopsies from right whales since the late 1980s (Brown *et al.* 1991). For the past two decades, long-term collaboration between photoidentification and genetic research teams has resulted in the collection of biopsy samples from over 75% of all photoidentified whales (Frasier *et al.* 2009). All biopsy samples have previously been profiled for sex (Shaw *et al.* 2003), profiled at a suite of 35 microsatellite loci (Frasier *et al.* 2006), and have been sequenced at the mitochondrial control region (Malik *et al.* 1999). The maintenance of a database containing these high-resolution genetic data enables linking right whale DNA extracted from fecal samples of unknown origin to previously genotyped individuals and their demographic and life history information (*e.g.,* Hamilton *et al.* 2007).

Genetic analysis of feces collected from terrestrial animals has become well established (Taberlet *et al.* 1996, 1997, Morin *et al.* 2001, Hedmark *et al.* 2004, Regnaut *et al.* 2006, Ball *et al.* 2007). However, to the best of our knowledge, collection and use of fecal material for genetic studies of marine mammals has been limited to the dugong (*Dugong dugon,* Tikel *et al.* 1996), bottlenose dolphins (*Tursiops truncatus*, Parsons 2001, Parsons *et al.* 1999, 2003, 2006), and right whales (Rolland *et al.* 2006, Gillett *et al.* 2008). In many studies, the ability to link fecal samples to known individuals is difficult as they are usually collected when no (Ernest *et al.* 2000, Miotto *et al.* 2007) or multiple (Parsons *et al*. 2003) animals are in the vicinity. Similarly, a majority of right whale fecal samples from this study were not collected from observed defecations, although some samples were associated with one or multiple individuals at the time of collection using photographic identification of individuals in the vicinity of sample collection. Therefore, molecular genetic identification was explored as a method to identify the source individual for specific fecal samples.

We recently demonstrated that DNA could be extracted from right whale fecal samples and developed a genetic profiling protocol for right whale fecal extracts (Gillett *et al.* 2008). As the majority of fecal samples contained low quantities and proportions of right whale DNA compared to other sources of DNA present in the extract, genotyping errors due to allelic dropout were a significant concern. To address this issue, a consensus profile was obtained from multiple amplifications of the same DNA extract. Although 35 microsatellite loci are currently being used for genetic profiling of biopsy samples from this species, profiling the fecal extracts at all loci was not feasible due to the time and cost associated with amplifying each sample multiple times at all loci. Therefore, five of the most variable microsatellite loci, a section of the mitochondrial control region, and molecular sex determination were chosen for profiling the fecal samples. The purpose of this study was to: (1) use this protocol for the molecular identification of fecal samples; (2) assess the extent to which the amount of right whale fecal DNA affects the reliability of sex determination, and the success in amplifying mitochondrial and microsatellite DNA, and the resolution of these genetic markers for individual identification; and (3) compare associations made with DNA to those made through photoidentification of individuals in the field.

Materials and Methods

Sample Collection

Between 1999 and 2005, right whale fecal samples were collected opportunistically during shipboard photoidentification surveys (Rolland *et al.* 2005, 2007*b*), from a dedicated fecal sampling vessel equipped with a scent detection dog (Rolland *et al.* 2006), and during necropsies of dead whales. Free-floating samples were gathered using a 300 μm nylon dip net (Sea-Gear Corp., Melbourne, FL, USA), drained of salt water and placed in polypropylene jars (without preservatives) on ice packs in a cooler until frozen at −20°C at the end of the day (Rolland *et al.* 2005, 2006). When possible, photographs were taken of the defecator, or, if defecation was not observed, of other individuals in the vicinity of sample collection. The standard procedure for photographing right whales for identification was used (Brown *et al.* 2007). Differing degrees of photoconfidence (PC) in associating the sample with a photographed whale were assigned at the time of collection. PC values were scored as PC 1: fecal samples had a definite association with one individual (*e.g.,* defecation

was observed or the sample was collected from a dead individual), PC 2: defecation was not observed, but fecal samples were associated with one to five whales in the vicinity of the sample using photographs taken at the time of collection, and PC 3: fecal samples were from an unknown whale (*e.g.,* defecation was not observed and no right whales were photographed in the immediate vicinity).

DNA Extraction, Quantification, and Genetic Profiling

Laboratory analyses were performed at the Natural Resources DNA Profiling and Forensics Centre (NRDPFC), and were subjected to stringent profiling standards and contamination controls. Extractions and PCRs were set up in areas free of amplified product, negative controls were included to screen for contamination, and sterile filter tips were used during all laboratory procedures. Fecal samples were extracted using a modified Qiagen DNeasy extraction protocol following Rolland *et al.* (2006). Right whale DNA was quantified following Gillett *et al.* (2008). Briefly, the total amount of DNA extracted was assessed using PicoGreen fluorescence enhancement (Molecular Probes, Ahn *et al.* 1996) and the amount of right whale DNA present in the extract was quantified using comparative amplifications of fecal extracts and known concentrations of right whale control DNA (Gillett *et al.* 2008). Right whale extracts were sequenced at a 218 bp variable segment of the mitochondrial control region following Rolland *et al.* (2006). Depending on the amount of right whale DNA available, each extract was profiled independently up to seven times at five of the most polymorphic microsatellite markers currently used to profile the right whale population (IGF1, GT023, Tex Vet17, Tex Vet20, RW4-17; Frasier *et al.* 2006) following the genotyping protocol developed in Gillett *et al.* (2008). A consensus profile was produced for each sample by combining results from each independent amplification, thereby addressing the problem of allelic dropout resulting from the low quantity of DNA template.

Sex was determined through coamplification of the *sry* and *ZFX* genes using the primers SRY-Y53-3C, SRY-Y53-3D (Fain and Lemay 1995), ZFX-P2-3EZ, and ZFX-P1-5EZ (Aasen and Medrano 1990). Each extract was amplified at least twice and results were only accepted if multiple amplifications resulted in the same sex. Females exhibited a single band of approximately 400 base pairs (bp), while males exhibited two bands of approximately 200 and 400 bp. Amplification consisted of a 15 μL reaction (0.3 μg/μL BSA, 1 × PCR Buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.3 μM of each primer, 0.1 U/μL *Taq* DNA Polymerase, and <0.05–>1 ng of functional genomic right whale DNA). The following cycling conditions were used for amplification: 94°C for 5 min; 50 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min; followed by a final extension of 60°C for 45 min. Amplified products were electrophoresed in a 1.5% agarose gel stained with ethidium bromide and run alongside a Low DNA Mass Ladder (Invitrogen) at 100 V for 45 min.

Photographic and Genetic Databases

Photographs taken of individual whales during fecal sample collection (1999 to 2005) were compared to all individuals in the *North Atlantic Right Whale Catalog* by researchers from the New England Aquarium following methods outlined in Kraus *et al.* (1986) and Hamilton *et al.* (2007). Composite genetic profiles obtained

from fecal samples were compared to previously obtained profiles from skin samples of known individuals housed in the *North Atlantic Right Whale Genetic Database*. Photoidentification data from 1980 to 2005 and all genetic data available for this species were used in these analyses. The error rates for these data sets have been reported previously as 0.0308 errors per photoidentification and 0.0327 errors per multilocus profile (Frasier *et al.* 2009). However, to date, no genotyping errors have been identified in any genetic profiles that were obtained from replicate sampling events of the same individual for any of the microsatellite loci used in this study.

Exclusionary Power

The probability of identity statistic, $P_{(ID)}$, was used to estimate the statistical power associated with identifying individuals from the seven markers used to profile the fecal samples (five microsatellite loci, sex, and mitochondrial haplotype). This statistic uses allele frequencies (under the assumption of Hardy–Weinberg equilibrium) to estimate the probability of randomly sampling two unrelated individuals that have identical multilocus genotypes (*e.g.,* Paetkau and Strobeck 1994, Woods *et al.* 1999). The simplest version of this statistic is calculated as p^2 for homozygotes or $2pq$ for heterozygotes, where p and q represent the frequency of the allele of interest. When multiple loci are used, the statistic is calculated by combining frequencies across all loci using the product rule (Li 1976). The $P_{(ID)}$ typically differs between individuals because it is calculated using the frequencies of the alleles present in the individuals genetic profile. Genetic profiles containing rare alleles are less likely to be present in two randomly chosen individuals in the population and result in a lower $P_{(ID)}$. Conversely, genetic profiles containing common alleles are more likely to be present in two randomly chosen individuals and result in a much higher $P_{(ID)}$.

Theoretical estimates for the $P_{(ID)}$ (of both the total population and for each individual profile) were calculated from allele frequencies obtained from genetic profiles of known individuals in the genetic database ($n = 382$) using the seven markers the fecal samples were profiled with. An assumption of the theoretical $P_{(ID)}$ is that all alleles are independent. However, in small populations alleles may not be independent because many individuals may be related, resulting in an underestimate of true probability of finding identical genetic profiles in the population (Taberlet and Luikart 1999, Waits *et al.* 2001). In order to account for the presence of relatives, the accuracy of the theoretical $P_{(ID)}$ for the population was examined by comparing it to the observed $P_{(ID)}$. The theoretical $P_{(ID)}$ was calculated following Paetkau and Strobeck (1994) using the equation $\sum p_i^4 + \sum\sum(2p_i * p_j)^2$; where p_i and p_j represent the frequencies of the ith and jth alleles, respectively, and where $i \neq j$. The observed $P_{(ID)}$ was calculated by taking genetic profiles of all known individuals at the seven markers used to profile the fecal samples, and determining the proportion of all possible pairs of individuals that had identical profiles at those loci ($n(n - 1)/2$; Waits *et al.* 2001, Bonin *et al.* 2004) using the identity check function in Cervus 2.0 (Marshall *et al.* 1998). To obtain an accurate assessment of the observed $P_{(ID)}$, individuals were removed if genotypes for less than six of the seven markers were available. To account for the presence of relatives in the sample set, the half-sib $P_{(ID)}$ was calculated using the five microsatellite markers and sex information following Evett and Weir (1998). The full-sib $P_{(ID)}$ was not calculated, as only one documented case of full-sibs in 90 paternities have been identified, and this relationship was, therefore, not applicable to this population (Frasier *et al.* 2007*a*).

Log-Likelihood Ratios

Log-likelihood ratios, incorporating sightings and genetic data, were developed to assess the confidence in the association of a whale with a particular fecal sample. When only one whale in the genetic database remained nonexcluded as the potential defecator, the probability that another nonprofiled individual was the defecator was determined. When multiple individuals remained nonexcluded to a sample with associated sightings data (PC 1 and 2), the probability that each individual was the defecator relative to each other or relative to another nonprofiled individual was determined. Five parameters (discussed below) were included in these ratios. As a majority of samples were collected in the Bay of Fundy (BOF), most of the parameters were estimated using data from this habitat area.

Prior probability—The prior probability parameter assessed the probability that the fecal sample came from any right whale in the BOF. Due to the extensive survey effort in this habitat area, it was assumed that most of the right whales entering the BOF were photographed within a season, and that the fecal sample was equally likely to have originated from any individual in a given year. On average 139 (range: 103–179) different right whales were sighted annually in the BOF during the study period (data from 2005 were not included because photoidentification analysis for sightings during that year is not yet complete). Therefore, the probability that the fecal sample came from any whale identified in the BOF in a given year was estimated as 0.7% (1/139).

Photoconfidence—The photoconfidence parameter assessed the probability that the photographed whale was the defecator. Estimates for this parameter were calculated from fecal samples that: (1) had one whale genetically nonexcluded as being the potential defecator, (2) contained >50 pg of right whale DNA per amplification, (3) had associated photographic data, and (4) had a low theoretical ($<1.0 \times 10^{-4}$; 1 pair of unrelated individuals in a population of 10,000 expected to share the same profile by chance) and half-sib $P_{(ID)}$ ($<5.0 \times 10^{-2}$; 1 pair of half-sibs in every 20 pairs expected to share the same profile by chance). These values were chosen as cut-offs as 1.0×10^{-4} should be sufficient to differentiate between individuals in a population of <400 individuals, and 5.0×10^{-2} should be sufficient to differentiate between half-sibs in this population as data based on known mother–calf pairs and inferred paternities (Frasier *et al.* 2007*a*) suggest that no individual has more than 20 documented half-sibs (unpublished data). The photoconfidence parameter was not included for PC 3 samples as photographs were not associated with these samples. Estimates varied depending on the associated PC rating. The probability that the photoidentified whale was the defecator of a PC 1 sample was 97% (100%−3% photoidentification error rate; Frasier *et al.* 2009). Comparatively, the probability that the photoidentified whale was the defecator for a PC 2 sample was 74% (97%−23%), as whales genetically associated with these samples were not photographed when the sample was collected for 23% of the events.

Molecular identification—The molecular identification parameter assessed the probability that another individual in the population had the same genetic profile as the associated whale. The probability that the complete fecal profile was from the associated whale was 99.4% (100%−0.6% profiling error/five loci biopsy genotypes; Frasier *et al.* 2009). The theoretical and half-sib $P_{(ID)}$ that was associated with the fecal profile was incorporated to account for the probability that another unprofiled individual (unrelated or related, respectively) had the same genetic profile. When multiple whales remained nonexcluded as the potential candidate,

the probability that each fecal profile was consistent with the associated whales was 99.4%.

DNA profiles—The DNA profile parameter assessed the probability that the defecator had been biopsied. As full genetic profiles were available from 80% of all whales identified in the BOF, the probability that the defecator had been biopsied was set at 80%. Similarly, when multiple whales remained nonexcluded the probability that each individual was biopsied was 80%.

Sightings—When multiple whales were nonexcluded from fecal samples with associated sightings data, a sightings parameter was used to determine the probability that the individual associated with the fecal sample through photoidentification analysis was more likely to be the defecator than the other nonexcluded individual(s). For this estimate the sightings history of whales from PC 1 samples collected in the BOF were assessed. Sightings for which the whale was seen defecating were not included in this estimate because all PC 1 individuals were seen at sample collection. Fifty-three percent of individuals associated with PC 1 samples were resighted on the day the sample was collected. However, as on any day, photoidentification teams attempt to document as many different individuals as possible, 53% is likely low due to inconsistent sampling effort. Therefore, for this parameter we used the probability that the defecator was seen within three sighting days of sample collection, where a sighting day consisted of any day the photoidentification team was surveying in the BOF and three sighting days consisted of any continuous 3-d period that included the day of sample collection. Seventy-one percent of individuals associated with PC 1 samples were resighted within this time frame.

Molecular Identification

Molecular identifications were made by comparing the composite sex and mitochondrial and microsatellite profiles for each fecal sample with all previously established genetic profiles. If a locus in the fecal profile could not be amplified for the recommended number of repetitions because of limited amounts of DNA (Gillett *et al.* 2008), and appeared to have only one allele, a hemizygous genotype (a genotype from which only one copy of the gene has been obtained) for that locus was accepted. During the exclusion analyses mismatches were only permitted at loci that were considered hemizygous. These loci were treated as both a homozygote for the identified allele and a heterozygote for the identified allele and a second unknown allele. When one whale in the genetic database was nonexcluded as being the potential defecator, all photographic identifications of the whale in all habitat areas were reviewed to determine where it had been sighted that year. For all analyses, whales that were dead or presumed dead (*e.g.,* not sighted for at least 6 yr; Knowlton *et al.* 1994, Hamilton *et al.* 2007) when the sample was collected were excluded as being the defecator.

RESULTS

Samples and Quantification of DNA

One hundred and eighteen samples (containing 20 PC 1, 38 PC 2, and 60 PC 3 samples) were used in this study. A majority of the samples were collected in the BOF ($n = 110$). The remaining samples were collected in other areas of the right

Table 1. Percentage of amplifications resulting in PCR product for mitochondrial (mtDNA), gender, and microsatellite (Tex Vet20, Tex Vet17, RW4-17, IGF1, and GT023) amplifications with respect to the amount of right whale template added.

DNA			Microsatellite loci					
(pg)/rxn	mtDNA	Gender	TexVet20	TexVet17	RW4-17	IGF1	GT023	Average
≤50	100%	52%	84%	80%	57%	36%	60%	64%
	65/65	59/114	194/230	201/251	96/169	87/239	109/182	687/1071
51–200	100%	82%	95%	95%	87%	67%	77%	83%
	17/17	27/33	87/92	70/74	47/53	67/100	62/81	333/400
201–500	100%	85%	100%	96%	80%	77%	79%	86%
	11/11	23/27	43/43	25/26	39/49	35/45	43/53	185/216
>500	100%	95%	98%	96%	76%	89%	90%	91%
	25/25	39/41	58/59	53/55	35/46	42/47	56/62	244/269
Average	100%	69%	90%	86%	68%	54%	71%	
	118/118	148/215	382/424	349/406	217/317	231/431	270/378	

whales range including: the Great South Channel ($n = 3$), Roseway Basin ($n = 1$), North Carolina ($n = 2$), Florida ($n = 1$), and Norway ($n = 1$). Samples from North Carolina, Florida, and one of the samples from the Great South Channel were taken from dead individuals during necropsies. Over 50% of the fecal extracts contained ≤10 pg/μL of functional right whale DNA ($n = 65/118$). Of the remaining samples, 17 contained 10–40 pg/μL, 11 contained 41–100 pg/μL, and 25 contained >100 pg/μL of right whale DNA (Table 1). The quantity of right whale template present in extracts from necropsied individuals varied from <10 pg/μL in the Great South Channel sample, 20 pg/μL and 200 pg/μL in the samples from North Carolina, and >1,000 pg/μL from the Florida sample. The Florida sample was collected a few hours after death and was taken directly from the colon of a neonate that stranded alive. The remaining samples were collected from whales in advanced states of decomposition from individuals that were found after they had already died.

Photographic and Genetic Databases

In total, 6,088 photographic records collected between 1999 and 2005 were analyzed. These records represented 388 known individuals, of which 80% (309/388) were sighted at least once in the BOF where a majority of fecal samples were collected. DNA profiles consisting of sex, mitochondrial control region haplotype, and 35 microsatellite loci were available for 382 known individuals. Of the right whales sighted in the BOF between 1999 and 2005, 80% ($n = 247$) were profiled.

DNA Profiling Strategy and Amplification Consistency

For the North Atlantic right whale population, comprising approximately 350 to 400 individuals, a theoretical $P_{(ID)}$ of 1×10^{-3} should be sufficient to differentiate between individuals, as less than one pair of unrelated individuals (4.0×10^{-1}, $400 \times P_{(ID)}$) in a population of 400 would be expected to share the same profile by chance. Additionally, for this species, a half-sib $P_{(ID)}$ of $<2.0 \times 10^{-2}$ should be sufficient, as data based on known mother–calf pairs and genetically inferred paternities suggest

that right whale families contain a maximum of 20 half-sibs (unpublished data), and at this value, less than one pair of half-sibs (4.0×10^{-1}, $20 \times P_{(ID)}$) would be expected to share the same profile by chance. The five microsatellite loci, sex, and mitochondrial haplotype resulted in a theoretical population $P_{(ID)}$ of 5.22×10^{-5} and a half-sib $P_{(ID)}$ of 1.7×10^{-2}. This indicates that full genetic profiles at these markers offer enough resolution to associate fecal samples with complete profiles back to their originator (as less than one pair of unrelated individuals (2.1×10^{-2}, $400 \times P_{(ID)}$) in a population of 400, and less than one pair of half-sibs (3.5×10^{-1}, $20 \times P_{(ID)}$) in a species that has 20 known half-sibs per family would be expected to share the same profile by chance). The observed $P_{(ID)}$ for this population at the loci used in this study was 1.4×10^{-5} (one in 70,500 pairwise comparisons of 376 individuals had identical genetic profiles), again indicating that this profiling strategy provides adequate resolution to associate the majority of fecal samples back to their originator.

The consistency of the microsatellite, sex, and mitochondrial amplifications was assessed by determining the number of amplifications resulting in PCR product from samples with varying amounts of right whale template (Table 1). The consistency of microsatellite genotyping was calculated from >1,900 PCRs across all template amounts. The percentage of amplifications resulting in PCR product varied across template amounts and microsatellite loci, ranging from 54% to 90%, with an average of 74%. In general, when ≤50 pg of right whale DNA was available in the reaction, the number of amplifications required increased by 20%–30%. The majority of reactions using Tex Vet17 and Tex Vet20 primers resulted in PCR product across all template DNA amounts (80%–100%), while the number of amplifications resulting in PCR product for RW4-17, IGF, and GT023 loci decreased from 87%, 67%, and 77% when >50 pg of right whale DNA was available to 57%, 36%, and 60% when ≤50 pg of right whale DNA was available. The sex reaction showed a similar pattern as the number of required amplifications resulting in PCR product decreased from 82% to 52% when amplifications contained >50 pg and ≤50 pg of right whale DNA, respectively. PCR amplifications at the mitochondrial control region were the most robust, as all amplifications resulted in PCR product.

Molecular Identification

Of the 118 samples profiled, 80 containing information for the mitochondrial control region and three or more microsatellite loci were carried through to the association analysis. All PC 1 samples were included as they were associated with the defecator through analyses of photographs taken at the time of sample collection. Of the samples carried through to the association analysis, 20 were collected from a dead whale ($n = 4$) or from an observed defecator ($n = 16$; PC 1), 27 were associated with one to five individuals that were photographed in the immediate vicinity of the sample collection (PC 2), and 33 were collected when no whales were in the vicinity (PC 3). Using the protocol described here 61 samples, including the three samples collected from necropsies in North Carolina and Florida, were associated with one individual in the genetic database. Forty of those samples had information for all seven markers (Table 2). The profiles of the remaining samples had differing degrees of genetic information available (Table 2). The majority of the samples with incomplete profiles came from samples from which <50 pg/μL of right whale DNA was added to each reaction. The profiles of 12 samples represented new individuals to the genetic database (15%). Seven locus profiles were available for 9 of the 12 samples, one

Table 2. Summary of fecal samples associated with one individual using genetic exclusions. The number of markers available for the association analysis, the number of hemizygous microsatellite loci in the profile (loci with partial information), and the range of the resulting theoretical and half-sib $P_{(ID)}$ statistics for those samples are indicated.

Number of samples	Total number of markers	Number of hemizygous markers	Theoretical $P_{(ID)}$	Half-sib $P_{(ID)}$
40	7	0	5.4×10^{-6}–9.0×10^{-14}	9.1×10^{-3}–3.8×10^{-5}
6	7	1	2.7×10^{-6}–3.7×10^{-10}	1.3×10^{-2}–3.2×10^{-4}
2	7	2	6.8×10^{-6}–2.0×10^{-8}	1.1×10^{-2}–1.3×10^{-2}
4	6	0	6.8×10^{-8}–1.2×10^{-8}	5.9×10^{-3}–2.3×10^{-3}
2	6	2	5.2×10^{-5}–2.3×10^{-8}	3.1×10^{-2}–7.7×10^{-3}
4	6	1	3.5×10^{-6}–3.3×10^{-12}	2.1×10^{-2}–1.3×10^{-3}
1	5	0	5.6×10^{-6}	1.8×10^{-2}
1	5	1	8.6×10^{-6}	2.0×10^{-2}
1	4	1	8.9×10^{-8}	7.5×10^{-3}

sample exhibited a four-locus profile and two samples exhibited a five-locus profile. Regardless of the fact that some samples had incomplete information, we can be confident that they represent new individuals to the genetic database because the profiles were not present in any individual currently archived. Multiple individuals remained nonexcluded as the potential defecator for the remaining samples ($n = 7$). Complete profiles were not obtained for these seven samples, resulting in decreased genetic resolution for linking these samples to the defecator.

When the defecator was photographically identified with a high degree of confidence (PC 1; $n = 20$), comparisons to the genetic database resulted in: (1) the defecator being the only whale nonexcluded from the sample ($n = 13$; includes the three necropsy samples from North Carolina and Florida), (2) all whales being excluded as the defecator (defecator profile was not available for comparison, $n = 3$), or (3) multiple individuals being associated with the sample with the known defecator being consistently one of the associated individuals ($n = 4$; includes the one necropsy sample collected from the Great South Channel) (Fig. 1). These data indicated that the profiles obtained from the right whale fecal extracts were reliably associating samples back to the defecator. Based on photoidentification data alone, log-likelihood ratios suggested that fecal samples from the PC 1category were 33 times more likely to have originated from the photographed individual than from another whale in the species ($\chi^2_{0.10-0.05,\,1} = 3.019$).

DNA profiles linked 24 of the 27 PC 2 samples back to known individuals. The remaining samples either represented a new individual to the genetic database ($n = 2$) or were associated with multiple individuals ($n = 1$). In 20 cases, genetic identifications supported associations made to one of the individuals photographed in the field (Fig. 1). Comparatively, DNA profiles were responsible for linking 24 of the 33 PC 3 samples back to known individuals, while seven represented new individuals to the genetic database and two were associated with multiple individuals in the genetic database (Fig. 1). All samples associated with multiple individuals were from samples with <50 pg/μL of right whale DNA per amplification and were not assigned because of decreased genetic resolution associated with incomplete profiles.

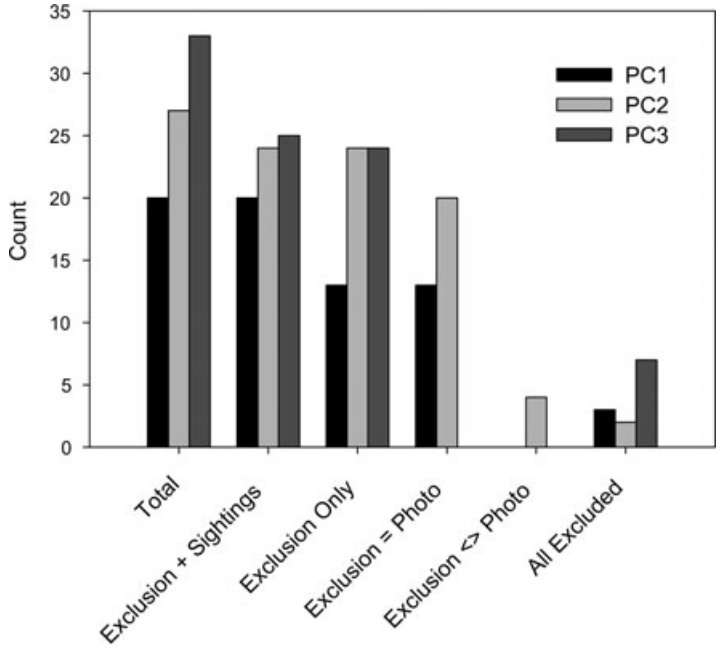

Figure 1. Summary of the outcome of the association analysis for all samples with respect to their photoconfidence level ($n = 20$ PC 1, $n = 27$ PC 2, $n = 33$ PC 3). Indicated are the total number of samples in each category (Total), the number of samples associated with one individual through a combination of sightings and genetic data (Exclusion + Sighting), the number of samples associated with a single individual if only genetic profiles were considered (Exclusion Only), the number samples where the individual photographed and the individual genetically nonexcluded were the same (Exclusion = Photo), the number of samples where the individual photographed and the individual genetically nonexcluded were different (Exclusion $\neq$ Photo), and the number of samples where all individuals in the genetic database were excluded as a potential defecator (All Excluded) are presented.

Individual Associations Using Molecular and Photographic Data

Sixty-nine fecal samples were linked back to a known individual with varying degrees of confidence using a combination of molecular and photographic data (Table 3). The theoretical and half-sib $P_{(ID)}$ for these samples ranged between 3.7×10^{-4}–9.0×10^{-14} and 1.2×10^{-1}–3.7×10^{-5}, respectively. In all but one case, log-likelihood ratios indicated that the fecal samples were at least 33 times more likely to have originated from the nonexcluded whale than from another nonprofiled whale in the population ($\chi^2_{\leq 0.05-0.10,1} \geq 3.645$; Table 3). These likelihood ratios were greater than those identified from PC 1 samples using only photoidentification data, and, therefore, suggest that there is a high probability that the whales nonexcluded from these samples were the defecators. In total, the 68 fecal samples represented 61 different whales. Samples were collected on multiple occasions from four individuals, either across 2 ($n = 3$), or 3 yr ($n = 1$). Genetic profiles from three PC 1 samples and nine PC 2 and 3 samples were not present in the genetic database. As none of the profiles were the same, these samples represented 12 new individuals to the

Table 3. Number of known right whales associated with each fecal sample ($n = 80$) using a combination of genetic exclusions and sightings data. The theoretical $P_{(ID)}$, half-sib $P_{(ID)}$, and likelihood that the sample come from the identified whale are presented. All samples had information for the mitochondrial control region and ≥3 microsatellite loci. Theoretical and half-sib $P_{(ID)}$ statistics were calculated following Paetkau and Strobeck (1994) and Evett and Weir (1998), respectively.

Number of whales	n	Theoretical $P_{(ID)}$	Half-sib $P_{(ID)}$	Log likelihood ratio	P
1	1	8.2×10^{-7}	1.2×10^{-1}	3.2×10^{1}:1	0.10–0.05
	10	3.7×10^{-4}–3.1×10^{-10}	1.1×10^{-1}–4.5×10^{-4}	3.3×10^{1}–5.4×10^{2}:1	0.10–0.05
	18	5.2×10^{-5}–5.8×10^{-9}	3.1×10^{-2}–3.1×10^{-3}	1.3×10^{2}–2.1×10^{3}:1	0.05–0.01
	40	1.5×10^{-7}–9.0×10^{-14}	3.8×10^{-3}–3.7×10^{-5}	2.3×10^{3}–3.3×10^{6}:1	<0.01
4	1	3.7×10^{-4}	5.0×10^{-2}	n/a	n/a
24	1	2.2×10^{-4}	5.9×10^{-2}	n/a	n/a
0	9	1.7×10^{-6}–9.35×10^{-9}	2.6×10^{-2}–1.0×10^{-3}	n/a	n/a

genetic database. Due to incomplete genetic profiles, one PC 2 and one PC 3 sample remained associated with multiple whales.

Comparison of Genetic Associations to Sightings Data

Not including PC 1 samples, individual whales that were the only genetically nonexcluded whale associated with a fecal sample ($n = 48$) were usually sighted in the same habitat area, and on the same day that the sample was collected ($n = 35/48$). Whales from the remaining associations were normally seen within 2 wk of the sample collection. Three associations did not fall into these categories. The first was a PC 2 sample collected in the BOF in 2003. This sample was associated with a whale that was born in 2002 and had only been sighted once in 2003 on the calving grounds when he was a yearling. Although this whale has been sighted 30 times since birth he has only been sighted three times in the BOF (once in 2004 and twice in 2005) over his entire sighting history (2002–2008). Five other individuals were photographed in the area when the sample was collected. Four whales were excluded as being the defecator, and although the remaining individual was not in the database, it was excluded because the mitochondrial haplotype of the whale's mother was different from that of the fecal sample. The second case was from a PC 3 sample that was collected in the BOF in 2001 and was associated with a whale that was seen in this habitat approximately 2 mo after the sample was collected. The final association was a PC 3 sample that was collected in the BOF in 2005. The associated whale was not seen in 2005 in the BOF, but was seen approximately 1 mo earlier in Roseway Basin and 1 mo later in the Gulf of Maine.

Discussion

Of 118 fecal samples, 80 resulted in DNA profiles that could be compared to profiles in the genetic database. These samples yielded 68 identifications, representing 61 different whales. Combined, these associations support ongoing fecal-based studies on reproductive status (Rolland *et al.* 2005, 2007*b*), assessment of stress (Hunt *et al.* 2006), parasite loads (Hughes-Hanks *et al.* 2005), and exposure to marine biotoxins (Doucette *et al.* 2006, Rolland *et al.* 2007*b*) so specific factors affecting the health and fitness of this species can be identified and evaluated. Associating fecal samples back to known whales and the ability of this noninvasive sampling method to recapture individuals between years allows us to (1) connect results obtained from fecal-based studies to long-term demographic, life history, and genetic databases, (2) supplement photoidentification data by filling in gaps in the sighting history of individuals who are more difficult to capture through photoidentification, and (3) estimate the proportion of whales missed during photoidentification surveys. Most importantly, associating fecal samples back to known individuals will allow us to monitor the health and reproductive status of specific individuals through time.

Exclusionary Power of Profiles

The seven markers used in this study resulted in a theoretical $P_{(ID)}$ of 5.2×10^{-5} and an observed $P_{(ID)}$ of 1.4×10^{-5}. The observed and theoretical values agreed well, thus providing sufficient genetic resolution to associate fecal samples with complete profiles to their originator. Although the theoretical and observed $P_{(ID)}$ for

right whales were similar in magnitude, the theoretical and observed $P_{(ID)}$ of other endangered species have been reported being up to three orders of magnitude different depending on the number of loci used, their variability, and the social structure of the species (Waits *et al.* 2001). Typically, as the number of loci considered increases (usually between 11 and 15 loci), the theoretical and observed $P_{(ID)}$ have been shown to approach the same value (Waits *et al.* 2001).

The theoretical and observed $P_{(ID)}$ for the North Atlantic right whale were very similar when a combination of five microsatellite loci, sex, and the mitochondrial haplotype were used, despite the fact that these whales come from a small population that exhibits extremely low levels of genetic diversity (Frasier *et al.* 2007*b*). Although fewer microsatellite loci were used in this study, screening fecal samples for sex and mitochondrial haplotype increased the genetic resolution. As North Atlantic right whales exhibit close to a 1:1 sex ratio (Hamilton *et al.* 1998), the addition of sex data functionally eliminated half of the population as a potential donor. Additionally, because only six mitochondrial haplotypes are present (Malik *et al.* 1999, McLeod and White 2009), 55%–99% of the population could be further excluded depending on the haplotype obtained. Therefore, profiling for sex and mitochondrial haplotype, in particular, were instrumental in the association analyses. For endangered species exhibiting low levels of genetic variation, this additional type of information may be more informative for linking individuals back to noninvasive samples than increasing the number of microsatellite loci.

Fecal DNA Profiles Not in the DNA Profile Database

All right whales in the genetic database were excluded as the potential defecator for 12 samples, indicating that they were collected from whales that had not previously been biopsied. This was expected, as 20% of all individuals sighted in the BOF during this study have not been biopsied and, therefore, some of these samples will be assigned to a known whale in the future as biopsy samples are obtained from additional whales. Three of these samples were collected from a whale seen defecating (PC 1), and in all cases, the whales associated with the sample were not available for comparison in the genetic database. The remaining nine samples were not associated with any whale at the time of collection.

Extensive annual survey efforts occur for this species in several habitat areas, and it is presumed that a majority of individuals have been photographed, because of the lack of new identifications (other than calves) in many years (Hamilton *et al.* 2007). Therefore, a direct count of the number of individuals presumed to be alive based on photoidentification data is thought to have provided the most accurate estimate of the current population size (Clapham *et al.* 1999). However, recent genetic analyses suggest that estimates based on direct counts of whales are an underestimation of the actual population size (Frasier *et al.* 2007*a*), indicating that some whales in the population have not been photoidentified. Because consistent, systematic population surveys have been conducted in the BOF since 1980 (Brown *et al.* 2007), new whales to the population that are not calves are rarely identified in this habitat area. Therefore, these samples most likely represent unbiopsied whales (although the possibility that some of these samples represent new individuals to the population cannot be ruled out). In less consistently surveyed right whale habitats (*e.g.,* the Great South Channel and Roseway Basin), new genetic profiles identified by fecal samples have a higher

probability of representing whales that have not previously been photographed, and, therefore, are not yet represented in current estimates of population size.

Associations to Individuals Not Sighted in the BOF

Although most whales identified from DNA profiles were also captured through photoidentification in the same habitat area and within 2 wk of sample collection ($n = 45/48$), a few individuals were not identified photographically. For example, right whale Eg #3279 was associated with a fecal sample collected in the BOF on 7 September 2003. This whale was born in 2002 and has been sighted 30 times since birth, but has only been sighted three times in the BOF (once in 2004 and twice in 2005). In 2003 this whale was only sighted once off the coast of Florida, when he was a yearling. Although this individual was not photographed in the BOF in 2003, his presence in this habitat was detectable *via* fecal genotyping. As 20% of the individuals sighted in the BOF were not present in the genetic database, it could be argued that this sample came from an unbiopsied individual. However, because the theoretical and half-sib $P_{(\mathrm{ID})}$ for the genetic profile of this sample were 1×10^{-11} and 1×10^{-4}, respectively, and the log-likelihood ratio for this sample was highly significant ($\chi^2_{\leq 0.001-0.005,\,1} \geq 8.255$), the probability of this occurring was low. Comparatively, right whale Eg #1817 was the only individual associated with a PC 3 sample that was collected in 2005. The theoretical and half-sib $P_{(\mathrm{ID})}$ for the genetic profile of this sample were 8.56×10^{-6} and 1.99×10^{-2}, respectively and the log-likelihood ratio for this sample was significant ($\chi^2_{\leq 0.025-0.05,\,1} \geq 4.581$). However, the profile was missing information for the microsatellite GT023 was hemizygous for Tex Vet17, and sex could not be resolved. Both of Eg #1817s documented offspring that were born before 2005 had been profiled and excluded as potential donors, but as she was identified at an unknown age in 1988, it is unknown who her half-sibs are in the population and, therefore, it may be possible that this sample came from a relative that has not yet been profiled. Additionally, fecal hormone data indicated that the fecal sample originated from a male, bringing this identification into question. Because of these concerns, additional loci should be screened in order to resolve this identification.

DNA Profiling Strategy and Amplification Consistency

Fecal samples with >50 pg of right whale DNA available per reaction amplified more consistently than samples containing less right whale DNA (Table 1). This resulted in incomplete profiles for the majority of samples for which <50 pg of right whale DNA could be added to the PCR, and therefore, limited genetic resolution for individual identification for the majority of fecal samples with lower template amounts. In order to maximize results from these samples, genetic resolution could be increased by the selective completion of genotypes with hemizygous data that are known to differentiate between the nonexcluded whales. Alternatively, additional loci that are more robust at lower template amounts could be optimized for use on fecal DNA or fecal extracts could be quantified to determine the probability of obtaining a full profile from that sample before other analyses on the fecal samples are undertaken.

Conclusions

The sex, mitochondrial, and microsatellite markers used in this study provided sufficient resolution for individual identification for samples where complete profiles were obtained. Using a combination of genetics and sightings data, 68 fecal samples were associated with known right whales, and 12 samples were identified that were new to the genetic database. Almost all of the identified whales were sighted within 2 wk of sample collection, indicating that the whales were in the BOF within a few weeks of the sample collection date. Three individuals were not seen in the BOF around the time the sample was collected, allowing us to fill gaps in the sighting history of those individuals through the collection of their feces. However, additional loci should be added to the genetic profile of these samples to confirm the identifications before data from these samples are used in subsequent analyses.

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