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Tracking mtDNA Heteroplasmy through Multiple Generations in the North Atlantic Right Whale (*Eubalaena glacialis*)

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Mitochondrial heteroplasmy has been identified in a variety of species and can result from either paternal leakage, whereby sperm mitochondria enter the ova during fertilization, or more commonly by the "survival" and proliferation of mutant variants within an organism. From an evolutionary perspective, this process represents the generation of new mitochondrial diversity within a species. Although this has been documented in some mammalian species, it has been reported from relatively few wild mammalian populations and in no wild nonhuman population has the transfer and segregation of mitochondrial heteroplasmy been tracked through multiple generations. We report on the first case of the identification and tracking of mitochondrial control region heteroplasmy through 3 generations in the North Atlantic right whale, *Eubalaena glacialis*. We also identify the full segregation to the mutant variant within a single generation and thus the development of a new haplotype (haplotype G) in a maternal lineage of this endangered species. Witnessed here is the generation of mitochondrial diversity in a genetically depauperate species.

Key words: *Eubalaena glacialis*, *control region*, *genetic diversity*, *matriline*, *mutation*, *paternal leakage*

Mitochondrial heteroplasmy (the presence of more than one mitochondrial variant within an organism) has been identified in a variety of species, with variants occurring in the form of point mutations and in varying numbers of tandem repeated sequence units (Aquadro and Greenberg 1983; Mignotte et al. 1990). Heteroplasmy can result from either paternal leakage, whereby sperm mitochondria enter the ova during fertilization, or more commonly by the "survival" and proliferation of mutant variants within an organism. Although heteroplasmy is a characteristic of all organisms, it is usually at such a low ratio of mutant variants that it is not detected using standard genetic analyses. Over time, most variants are lost to genetic drift, but more rarely, these variants increase in frequency to a detectable level and become the dominant mitochondrial variant. From an evolutionary perspective, this process represents the generation of new mitochondrial diversity within a population.

Presence and degree of heteroplasmy within an organism can vary across body tissues and is determined by random genetic drift. For example, an individual may have some degree of heteroplasmy within the skin or hair, whereas other areas appear fixed for a single variant (i.e., the germ line) and vice versa. As a result, transmission of heteroplasmy to offspring will vary depending on when, and in what cells, the heteroplasmy originated and/or proliferated; a characteristic that can make the identification and tracking of heteroplasmy through maternal lineages challenging. It has been proposed that the probability of survival of mutant variants in the germ line is highest during the mitochondrial "bottleneck" that occurs just after fertilization (Lightowlers et al. 1997; Chinnery et al. 2000). During such a bottleneck event, random genetic drift of variants can lead to haplotypic differences between oocytes of a single individual and therefore haplotypic segregation among offspring. The rate and extent to which offspring haplotypes (and the relative proportions of particular variants) change between generations has been shown to vary extensively (Ashley et al. 1989; Wilson et al. 1997), with full segregation to the variant form occurring within as few as 1–3 generations (e.g., cows [Ashley et al. 1989; Koehler et al. 1991]).

Although mitochondrial heteroplasmy has been documented in some mammalian species (e.g., humans [Howell et al. 1992; Wilson et al. 1997; Huhne et al. 1998]; horse [Xu and Arnason 1994]; and cows [Hauswirth and Laipis 1982;

Ashley et al. 1989]), it has been reported from relatively few wild mammalian populations (i.e., evening bats [*Nycticeius humeralis*] [Wilkinson and Chapman 1991], South American camelids [*Camelidae* sp.] [Maté et al. 2007], rabbits and hares [*Leporidae* sp.] [Mignotte et al. 1990; Biju-Duval et al. 1991], elephant seals [*Mirounga* spp.] [Hoelzel et al. 1993], humpback whales [*Megaptera novaeangliae*] [Baker et al. 1990], and sperm whales [*Physeter macrocephalus*] [Lyrholm and Gyllensten 1998]). However, to our knowledge there is no wild population in which the transfer and segregation of mitochondrial heteroplasmy has been tracked through multiple generations. Here, we report the first case of the identification and tracking of mitochondrial control region heteroplasmy through multiple generations in a wild mammalian population; the North Atlantic right whale (*Eubalaena glacialis*). We also identify the full segregation to the mutant variant form within a single generation and the generation of a new haplotype in a maternal lineage of this endangered species.

Materials and Methods

Heteroplasmy of the mitochondrial control region was detected in 6 individual right whales during sequencing of the mitochondrial control region as a part of ongoing research to evaluate population genetics of the North Atlantic right whale in the western North Atlantic (reviewed by Frasier, McLeod et al. 2007). Skin biopsies were originally collected from free ranging animals between 1988 and 2008. DNA was extracted from these samples as per Shaw et al. (2003) and Wang et al. (2008).

A 487 bp fragment of the mitochondrial control region was amplified using the primers BMYUP098 (Rastogi et al. 2004) and LP585 (Malik et al. 2000). Polymerase chain reaction (PCR) cocktail conditions were as follows within a 20 μl reaction volume containing 5 ng DNA: 1× PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl) (Invitrogen, Burlington, ON), 2 mM $MgCl_2$ (Invitrogen), 0.2 mM each dNTP (Amersham Biosciences, Piscataway, NJ), 0.1 μg/μl bovine serum albumin (Sigma, Oakville, ON), 0.05 U/μl *Taq* polymerase (Invitrogen), and 0.3 μM of each primer. Cycling conditions were as follows: 5 min denaturation at 94 °C; 30 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; followed by a final extension step at 65 °C for 45 min. PCR product was then evaluated and purified as per McLeod et al. (2008) and sequenced (using primer LP585) on a MegaBace 1000 DNA Analysis System (GE Healthcare, Piscataway, NJ).

All mitochondrial sequences were edited and aligned by eye and then assigned haplotypes by comparing them to all known North Atlantic right whale haplotypes (Malik et al. 2000; Rastogi et al. 2004) (haplotype variable sites shown in Supplementary Table S1). To confirm "new" or heteroplasmic haplotypes (identified by 2 bases present at a single sequence site in the sequence electropherograms), samples were reamplified, purified, and sequenced in the reverse direction using the primer BMYUP098. As well, samples were cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen) with a TA plasmid vector (pCR4-TOPO). Inserts were verified by amplification (with primers LP585 and BMYUP098) following the protocol above. Inserts of the correct size were then sequenced as per McLeod et al. (2008). Positive and negative (blank) controls were included during all steps of the laboratory processing and analysis (i.e., extraction, amplification, purification, sequencing, and cloning).

To assess the genetic relationships between individuals, we evaluated photo-identification and genetics data available from the North Atlantic right whale Consortium Databases (Right Whale Consortium 2008); databases containing data from more than 20 years of research on the species (Kraus and Rolland 2007).

Results

A single base-pair mitochondrial control region heteroplasmy (nucleotides A and G) was identified at position 158 (after Malik et al. 2000) in 6 individual right whales (XXX13, [New England Aquarium] NEA 1411, NEA 1123, NEA 3123, NEA 2123, and NEA 3323). Heteroplasmy was then confirmed by sequencing in the reverse direction, sequencing DNA extracted from any other samples collected from these individuals (where available), and through cloning. Six clones were sequenced for heteroplasmic individuals.

Through further assessment of the relationships of heteroplasmic individuals, it was identified that 5 of the individuals are part of a single matriline of a nonheteroplasmic female (NEA 1142) (Figure 1; Supplementary Figures S1–S10) (Right Whale Consortium 2008). Mother–calf associations are designated using both observational and genetic data. For a calf to be recognized as the offspring of a particular female through observation only, the 2 must be seen in association multiple times during the calf's first year. This has been confirmed for all individuals in the lineage (with the exception of NEA 1142 and her calf of 1977 which were seen together once). These associations are later confirmed through genetic profiling of both individuals (this has been confirmed for the following calves in the lineage: NEA 1411, NEA 1123, NEA 3442, NEA 3143, NEA 2042, NEA 2642, NEA 3123, and NEA 3423). The remaining heteroplasmic individual (XXX13) died in 2002, and although familial relationships to this animal are not known, it is likely that it was a part of this matriline.

The matriarch of the matriline (NEA 1142) has given birth to 8 calves (NEA 1411, NEA 1123, NEA 3442, NEA 3142, NEA 2042, NEA 2642, and unidentified calves from 1977 to 2009) (Right Whale Consortium 2008). Two of these calves are heteroplasmic (NEA 1411 and NEA 1123), 4 share the same haplotype as their mother (haplotype "A"; GenBank accession AF395039), whereas the haplotypes of the remaining 2 have yet been identified. One of these calves (NEA 1123) has given birth to 4 calves (Right Whale Consortium 2008), 2 of which are heteroplasmic (NEA 3123 and NEA 2123), 1 of which (NEA 3423) is

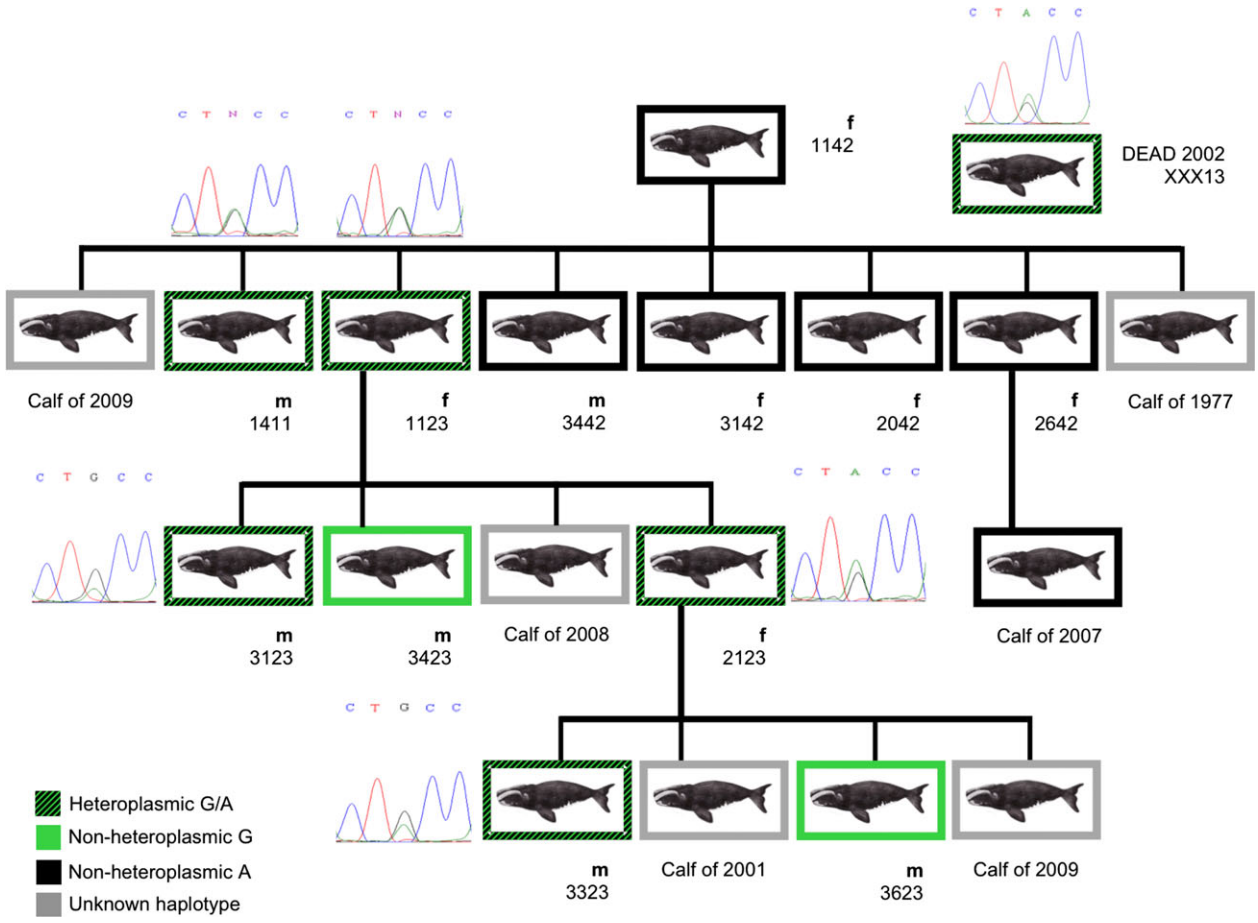

Figure 1. Matriline of North Atlantic right whale NEA 1142. Numbers by each box are the NEA individual identification numbers. Boxes bordered in green, black, green/black striping, and gray show individuals with mitochondrial control region haplotypes that are: nonheteroplasmic (A), nonheteroplasmic (G), heteroplasmic (A/G), and unknown, respectively. Individuals have unknown haplotypes because samples have not been collected (NEA 1142's calf of 1977 and NEA 2123's calf of 2001) or have not yet been submitted for DNA analysis (NEA 1142's calf of 2009 and 1123's calf of 2008). Males and females are identified by "m" and "f," respectively. Electropherograms of heteroplasmic individuals are also shown. For additional images of electropherograms see Supplementary Figures S1–S10.

homoplasmic for the new variant identified within the heteroplasmic individuals (haplotype "G"; GenBank accession GQ927329), and 1 of which has not yet been genetically assessed. One of these heteroplasmic calves (NEA 2123) has given birth to 4 calves (Right Whale Consortium 2008), 2 of which have not been genetically assessed (Calf of 2001 and the Calf of 2009), 1 of which is heteroplasmic (NEA 3323), and 1 which is the new variant "G" (NEA 3623). Thus, rapid segregation of the mutant variant (within 1–2 generations) has resulted in a new haplotype (haplotype "G") for the population within 2 individuals. Figure 1 illustrates what is currently known of the sexes, haplotypes, and relationships of individuals that have been identified within this matriline (Right Whale Consortium 2008).

To assess whether the heteroplasmy identified within individuals of the lineage is a result of paternal leakage during their conception, mitochondrial haplotypes for any currently identified paternities within the matriline were assessed. To date, 7 paternities have been genetically identified within this lineage (Frasier, Hamilton et al. 2007) and of the 2 known paternities that are associated with a heteroplasmic calf (NEA 1123 and NEA 3123), the mutant variant is not present in the father (Right Whale Consortium 2008). Additionally, the "new" variant is not a previously identified haplotype and cannot be from paternal leakage from any males currently within the genetic database, which contains ~80% of all individuals ever identified.

Discussion

Mitochondrial control region heteroplasmy was identified within a single matriline of the North Atlantic right whale. Analysis of this lineage shows that haplotype segregation has

occurred rapidly and in different directions (i.e., resulting in different haplotypes across offspring). As well, full segregation to the mutant variant has occurred in 2 individuals within 1–2 generations, with the establishment of a "new" haplotype in the population. Such rapid segregation has been illustrated previously in Holstein cattle (Ashley et al. 1989; Koehler et al. 1991), where segregation to a mutant variant has been shown to occur within 1–3 generations but has not (to our knowledge) been shown within any nonhuman wild mammalian population. This rapid segregation of haplotypes is thought to occur during the genetic "bottleneck" that occurs at an early stage in oogenesis (likely between the development stages of the primordial germ cells and the primary oocytes) (Hauswirth and Laipis 1982; Chinnery et al. 2000; reviewed by Lightowlers et al. 1997). During this bottleneck, the number of mitochondrial DNA (mtDNA) present in the oogonia is small, and therefore, the effects of random genetic drift will be stronger. The increased effects of drift can result in more dramatic changes in the allele frequencies of rare mutants and potentially the rapid removal or fixation of mutant variants (Jenuth et al. 1996). Although this bottleneck has been estimated in some species (e.g., Jenuth et al. 1996), it is unknown for this species.

It is surprising that 2 offspring of a homoplasmic female appear to be spontaneous mutations to heteroplasmy. We consider it more likely that somewhere within her body (i.e., oocytes or other body tissues [e.g., Hauswirth et al. 1984; Wilson et al. 1997]) some degree of heteroplasmy exists at a level not detectable through sequencing of DNA extracted from skin cells (as done here). Consequently, it is also possible that any other seemingly homoplasmic "A" offspring within the matriline also carry some degree of undetected heteroplasmy. They too could produce offspring that appear to be heteroplasmic (A/G) or homoplasmic ("A" or "G"). This leads us to question whether there might be other heteroplasmic individuals or lineages that have not yet been identified. As a part of updating the genetic database for the North Atlantic right whale, any new samples as well as any samples previously typed using SSCP technology (i.e., Malik et al. 1999, 2000) are being sequenced. To date, 61% of individuals sampled have been haplotyped through sequencing and no other heteroplasmic matrilines have been identified. This possibility will be addressed through continued assessment of the haplotypes of new individuals added to the genetic database in the future.

The heteroplasmy observed within the population is most likely a result of either a spontaneous mtDNA mutation event or paternal mtDNA leakage (from a male carrying the "G" variant) that occurred either during the conception and development of the matriarch or within another female earlier in the lineage. The heteroplasmy is not likely a result of "recent" paternal leakage or spontaneous mutation events (i.e., during the conception of any offspring shown in Figure 1). Paternal leakage is unlikely because the mutant haplotype (haplotype "G") has not been identified previously in the population, even though ~80% of all individuals ever identified have been sampled and haplotyped (this study, Malik et al. 1999, 2000). Also, the fathers of 2 of the heteroplasmic calves have control region haplotypes that have previously been identified within the population. Finally, for the observed heteroplasmy to have been a result of a recent spontaneous mutation, this mutation would have had to have occurred at least 2 times.

There is always potential for error in sampling identity, however, we suggest that the approaches used in here to identify individuals has previously been shown to be reliable. Recently, error rates for both the photo-identification data (>25 years of data) and the high-resolution genetic profiles (>75% of the individuals in the photo-identification catalog) were estimated for this species (Frasier et al. 2009). It was estimated that the photo-identification data had 0.0308 errors/identification and the genetic data had 0.00121 errors/locus and 0.0327 errors/multilocus. These estimates, though not negligible, are considered "among the lowest error rates yet reported" (Frasier et al. 2009).

Here, we are witnessing first-hand the survival of a new mutation and the increase in genetic diversity in a genetically depauperate endangered species (Malik et al. 2000; Waldick et al. 2002). Although the 2 apparently homoplasmic individuals are males and so will not contribute the novel haplotype to future generations; the lineage now contains at least 2 heteroplasmic females and so there is still potential for future maintenance and segregation of the new variant. This study highlights the importance and value of long-term studies of wild populations. It is the long-term nature of research on the North Atlantic right whale that has facilitated the tracking of this single mutation through multiple generations in a long-lived species.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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