Delineating Patterns of Male Reproductive Success in the Western Gray Whale (*Eschrichtius robustus*) Population

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ABSTRACT

Monitoring of the critically endangered western gray whale population on its primary feeding ground off Sakhalin Island, Russia, has led to the collection of genetic samples from 83% of animals photographically identified from this area. The high proportion of sampled animals provides a rare opportunity to learn more about the mating system of a baleen whale species as well as to identify factors potentially influencing this small population's recovery. Utilizing a panel of 13 microsatellite loci, paternity analysis was conducted to identify breeding males and assess the distribution of male reproductive success. Using biopsy samples from 57 mother-calf pairs and 42 candidate males, putative fathers were identified for 46 to 53% of calves sampled in the population between 1995 and 2007. Although most males were assigned paternity of only one calf, a mild skew in the distribution of reproductive success was identified, with some males siring three to four calves over the 12 seasons of the study. Eighteen putative fathers were identified, and analysis of relatedness patterns among those calves which were not assigned a father suggested that an additional 15 males may be contributing to reproduction in the population. The relatively low success rate of the paternity analysis, in comparison to expectations derived from the percentage of photographically identified animals which have been sampled, indicates that some reproductive males may not use the Sakhalin feeding area on a regular basis. While the high percentage of "missing fathers" in this small population is puzzling, these results provide evidence that many of the animals identified on the Sakhalin feeding ground interbreed, presumably while sharing a common migratory route.

INTRODUCTION

Little is known about the mating systems of most species of baleen whales. Differences in the reproductive cycles of males and females suggest that mysticetes, like the majority of mammals, are polygynous (Clutton-Brock 1989, Mesnick and Ralls 2009). In most baleen whale species, females are restricted to producing a calf every two to three years due to the energetic constraints imposed by gestation and lactation, while males have potential reproductive rates that are much higher. These differences result in a skew in the number of reproductively available females relative to reproductively active males (*i.e.*, the operational sex ratio), increasing the extent to which males must compete for females and permitting variance in male reproductive success to develop. As has been proposed for aquatically mating pinnipeds (Bartholomew 1970), however, the degree of polygyny which can be established in baleen whales is likely

limited by the marine environment in which they breed, as females are highly mobile and resources are difficult to defend.

Much of the information available on mysticete mating systems has been derived from behavioral observations and the collection of physiological data. In humpback whales (*Megaptera novaeangliae*), the use of alternative mating tactics by males, including physical competition for access to females as well as "escorting" of females (Pack *et al.* 1998, Clapham 1996), has been documented and suggests a mechanism by which differential reproductive success might be generated in this species. In North Atlantic right whales (*Eubalaena glacialis*), males aggregate in large active groups and appear to compete for access to a female (Kraus and Hatch 2001). In addition, right whales, along with bowhead and gray whales, have high testes-to-body weight ratios, indicating that sperm competition is an important strategy utilized by males (Brownell and Ralls 1986). The existence of both pre- and post-copulatory competition for fertilization in this species suggest that male reproductive success may not be evenly distributed across individuals.

While these studies have proven valuable in elucidating male mating strategies, they do not provide information on which males in a population successfully breed or how male reproductive success is allocated among individuals, which can have important implications for the maintenance of genetic diversity in small populations. The effective size of a population is determined not only by the number of animals contributing to successive generations, but also by the distribution of reproductive success among those individuals. High variance in reproductive success acts to decrease the effective size of the population (Hedrick 2005), thereby increasing the rate at which genetic diversity is lost. In addition, identifying reproductive pairs within populations can increase our understanding of the role that mate choice may play in inbreeding avoidance (*e.g.,* Archie *et al.* 2007) as well as of the relationship between offspring fitness and parental relatedness (*e.g.,* Amos *et al.* 2001).

Genetic analysis of paternity has the potential to provide detailed information on the reproductive success of individual males. In baleen whales, these analyses have thus far been limited to humpback (Clapham and Palsboll 1997, Nielsen *et al.* 2001, Cerchio *et al.* 2005) and North Atlantic right whales (Frasier *et al.* 2007). In studies of both species, the genetic results contributed valuable insight into patterns of reproduction which were not readily apparent based on previous studies. In humpback whales, the distribution of male reproductive success deviated from that predicted under random mating, but the variation was lower than expected given the apparent skew in the operational sex ratio and the degree of male competition observed (Cerchio *et al.* 2005). In North Atlantic right whales, males demonstrated high variance in reproductive success when compared to other aquatically-mating marine mammals, although the variance was low relative to those breeding terrestrially (Frasier *et al.* 2007). These results support the idea that sperm competition creates differential reproductive success among males, but that the lack of control over resources and mates in the aquatic environment limits the degree of polygyny which could develop (Frasier *et al.* 2007). Results from this study also suggested that the low level of genetic variability in this species may be influencing reproductive success. Calves for which both parents were identified had significantly higher levels of genetic diversity than expected under random mating, indicating that successful mating only occurs between individuals that are genetically dissimilar (Frasier 2005).

In gray whales, behavioral observations of multiple males mating with a female suggest a polygynous or promiscuous mating system (Jones and Swartz 1984). While mating behavior has been observed in all seasons, fertilization is thought to primarily occur during the southbound migration (Rice and Wolman 1971). Information from fetal growth rates suggests that females come into estrus during an approximately three-week period extending from late November to early December at the start of the southbound migration. Most females are thought to conceive during this period, although some females may ovulate approximately 40 days later when on or near the wintering grounds. Increased testes weight and the larger seminiferous tubules of males on the southbound migration, as compared to those of northbound and summer feeding males, also support a peak in spermatogenic activity in late autumn to early winter (Rice and Wolman 1971).

As aforementioned, the high testes weight to body weight ratios found in gray whales suggest that this species utilizes sperm competition as a strategy for males to obtain successful fertilizations (Brownell and Ralls 1986), raising the possibility that, like North Atlantic right whales, some variance in male

reproductive success may exist. Genetic paternity analysis would provide information valuable in assessing this possibility. Such a study would be difficult to conduct in the eastern gray whale population, given its large size and the relatively small proportion of animals which have been sampled. However, the population of gray whales found in the western North Pacific, which is both small and well-sampled, presents a valuable opportunity to conduct such an analysis. This population of whales was nearly extirpated by commercial whaling, which continued through at least 1966 (Brownell and Chun 1977, Weller *et al.* 2002). Unlike its eastern counterpart, which currently numbers approximately 22,000 animals (Punt and Wade 2010), the western population has remained severely depleted and is estimated to contain approximately 130 individuals of one year or older (Cooke *et al.* 2008). Western gray whales are listed as Critically Endangered by the IUCN (Baillie *et al.* 2004). Anthropogenic threats potentially jeopardizing the population's recovery include extensive oil and gas development on the population's primary feeding area (Weller *et al.* 2002, Reeves *et al.* 2005) and mortality of whales associated with entrapment in fishing gear during their migration past Japan (Brownell *et al.* 2007).

Much of what is known about this small population of whales has been derived from long-term studies on their primary feeding ground located in the coastal waters of northeastern Sakhalin Island, Russia. Following a pilot study in the summer of 1995, a monitoring program was initiated in 1997 and continues to date. Extensive photo-identification records collected as part of this effort have shown that western gray whales exhibit a high degree of seasonal site fidelity to the Sakhalin feeding area (Weller *et al.* 1999, 2008a). This site fidelity, in combination with the population's small size, has facilitated the collection of genetic samples from a high percentage (~83%) of animals identified on the feeding ground. Analysis of these samples using both mitochondrial DNA (mtDNA) and a panel of microsatellite markers has shown that the western population is genetically distinct from the much larger eastern gray whale population (LeDuc *et al.* 2002, Lang *et al.* 2010).

Given the high percentage of sampled animals and the availability of extensive sighting records for most individuals, genetic analysis of paternity in the western gray whale population will not only contribute to our understanding of mysticete mating systems but may also provide information important in assessing factors influencing the population's recovery. The low number of known reproductive females (n=24 between 1995 and 2007) has raised concern for the population's capacity for growth and recovery (Weller *et al.* 2002, 2008a). Little has been known, however, about the number of males which are breeding in the population, or how reproductive success is distributed among these animals. Although previous studies have suggested that the western gray whale population has retained relatively high levels of genetic diversity (Lang *et al.* 2010), information about factors potentially influencing the effective size of the population may provide insight into the past and future maintenance of genetic diversity in this population.

In addition, limited information is available about the movements of western gray whales outside of their feeding range. The wintering ground(s) for this population is suspected to be off the southern coast of China, but the location has yet to be confirmed. Sightings, strandings, and entrapments suggest that gray whales migrate along both the eastern and western coasts of Japan, as well as along mainland Asia (Brownell *et al.* 2008). Although photo-identification records have identified one of the animals entrapped off the Pacific coast of Japan as an animal known to utilize the Sakhalin feeding ground (Weller *et al.* 2008b), thus far this is the only established link between a feeding area and a migratory pathway. Given that mating is thought to occur primarily while on migratory routes, the use of paternity analysis to identify pairs of interbreeding animals will provide information on which animals have utilized the same areas for migration and potentially overwintering.

Finally, questions have been raised about the isolation of this small population. Although the eastern and western populations have traditionally been considered geographically separate, in recent years gray whales have been sighted in feeding areas located off the eastern coast of Kamchatka. Some of these whales are known to have visited the Sakhalin feeding area, while others are of unknown origin (Tyurneva *et al.* 2009). These sightings have raised speculation about possible overlap among feeding regions for the eastern and western populations. In addition, although genetic studies have confirmed that eastern and western gray whale populations are distinct, the level of nuclear differentiation between the two populations is relatively low, suggesting that some intermixing of eastern and western animals could be occurring (Lang *et al.* 2010). Such intermixing could entail the use of the Sakhalin feeding area by eastern

whales which then return to the eastern Pacific, or it might include some limited amount of interbreeding between the two populations. Although the lack of extensive sampling of the eastern population limits comparisons across populations, the proportion of reproduction that can be attributed to animals sampled off Sakhalin will provide some insight into the extent and nature of any interbreeding which may be occurring.

Between 1995 and 2007, 57 mother-calf pairs and 42 males of unknown age were sampled on the feeding ground off Sakhalin. These samples represent 90% of identified mother-calf pairs, and 83% (n=142) of all animals identified on the Sakhalin feeding ground during the study period. Using these samples and a suite of 13 microsatellite markers, a genetic paternity assessment was conducted for this population. The primary objectives of this work were to identify the number of males contributing to reproduction in the western population and to evaluate the distribution of reproductive success among these males. The results of the assessment not only expand our knowledge of mating systems of baleen whales, but also increase our understanding of factors potentially affecting the recovery of the western gray whale.

METHODS

Sample collection

Following a Russian-American pilot effort in 1995, a collaborative Russia-U.S. research program was established in 1997 which focuses on individual monitoring of western gray whales using photo-identification and genetic sampling (Weller *et al*., 1999, 2002). Field studies are carried out annually during summer months on the primary feeding ground off the northeastern coast of Sakhalin Island, Russia. Surveys (n=337) of this area have led to the photographic identification of 169 whales; genetic samples for 141 (83.4%) of the identified whales have been collected through biopsy-darting (Weller *et al.* 2008).

The genetic sample set includes samples collected from 57 animals which were first identified as calves (approximately 6-8 months of age) on the feeding ground and which are linked to known and genetically sampled mothers. Animals were identified as calves based on their small body size (approximately one third that of an adult), and, in most cases, their constant affiliation with a particular adult whale (Weller *et al.* 1999). For 54 of the calves, identification of the mother was first established via behavioral observations and confirmed by genetic analysis. Three calves were already separated from their mothers when first identified; in these cases the mother was identified using genetic parentage analysis alone.

The sex of all animals was determined using molecular methods (detailed in Lang *et al*. 2010). Forty-two animals were identified as males of unknown age and were included as candidate fathers in the analysis. Males first identified as calves which may have reached sexual maturity during the study period were also included as potential candidate males. Although it is not known at what age male western gray whales first reproduce, the earliest estimates of age at sexual maturity in eastern gray whales is five years (IWC, 1993). Therefore, male calves were included as potential candidates for those years in which they would have been at least five years of age and potentially capable of reproduction in the preceding season when fertilization would have occurred. To incorporate the changing number of candidates as calves from earlier years of the study became potentially sexually mature, paternity analysis was run separately for each year of the study.

Analysis

Samples utilized in the study had been genotyped at 13 microsatellite loci for use in the previous study; the protocols used to produce this data are detailed in Lang *et al*. 2010. Paternity was assessed using the likelihood-based approach as implemented in CERVUS v3.0 (Marshall *et al.* 1998, Kalinowski *et al.* 2007). The objective of this analysis was to identify the candidate father which was statistically the most likely to be the true parent of the calf. The likelihood for each candidate was calculated using information on the allele frequencies in the population, such that a candidate male which shared a rare allele at a given locus with the calf was considered to be more likely to be the true father than a candidate sharing a common allele with the calf. The number of mismatches between the genotype of the father and the genotype of the calf (after removal of the maternal contribution to the calf's genotype) was also utilized in the likelihood analysis.

Simulations were used to generate critical values allowing confidence in the assignments to be evaluated. These simulations used the allele frequency data from the population being analyzed to generate simulated genotypes for parent-offspring pairs and unrelated individuals. The simulated data were then used to calculate the likelihood of parentage for the true parent as well as for each of the unrelated candidate parents for the simulated offspring. For each candidate identified as the most likely parent (whether or not it represented the true parent), the difference in likelihood scores between that individual and the next most likely individual, referred to as Δ , was recorded. The distribution of Δ scores where the identified parent was the true parent with the distribution of scores where the identified parent was an unrelated individual was then compared to determine a critical value, such that the identified Δ was sufficiently large to distinguish true parents from unrelated parents at a specified (e.g. 95%) level of confidence.

One of the strengths of the CERVUS analysis is that it is able to account for potential errors in the dataset. Genotyping errors, mutations, or null alleles may create mismatches between the genotypes of candidate fathers and offspring, such that the true father might be falsely excluded when such factors are not taken into account. By incorporating an estimated error rate into the simulations run by CERVUS, the potential for such false exclusions is reduced. Here we ran the CERVUS analysis utilizing two different error rates. The first analysis assumed that no errors were present in the dataset, but this differs from a strict exclusionary approach in that likelihood scores were used to differentiate between candidate parents when more than one male had genotypes which matched that of the mother-calf dyad at all loci. The second analysis utilized an error rate of 0.01, allowing candidates to be assigned as putative fathers which had genotypes mismatching that of the mother-calf dyad at up to two loci. Additional parameters used in the simulations included 1) the number of simulated genotypes = 10,000, 2) proportion of loci typed = 0.99 (the true proportion), 3) minimum number of loci typed = 12, and 4) proportion of candidate males sampled=0.50. Given that the error rate and the proportion of sampled males are not known, additional simulations to explore the effects that these parameters may have had on the results are described in the Supplementary Information. Results were evaluated at both the strict (95%) and relaxed (80%) levels of statistical confidence. The allele frequencies utilized for the likelihood calculations and simulations incorporated only the genotypes of non-calves, to avoid skewing the allele frequency distribution by inclusion of known relatives.

The pool of candidate males changed during some years of the study, as males first identified as calves and known to be $\geq$ 5 years of age became incorporated in the analysis. As such, the simulations used to calculate critical values were run separately for years with different pools of candidate males, such that while the proportion of candidate males sampled (0.50) was held constant throughout the study, the number of candidates was updated to reflect the changing number of sampled males. The total number of candidate males used in the CERVUS simulation was set by multiplying the number of sampled males by two in accordance with the assumption that 50% of candidates had been sampled.

To provide insight into the reproductive success of unsampled males, the program DADSHARE (www.zoo.cam.ac.uk/zoostaff/amos, see Hoffman *et al.* 2003) was used to estimate the number of putative sires for the calves for which all sampled candidates were excluded. This program inferred paternal sibships by calculating pairwise paternal relatedness coefficients among unassigned offspring using the relatedness coefficient of Queller and Goodnight (1989). A clustering algorithm (UPGMA) was then used to produce a dendrogram linking the most closely related individuals and sorting offspring into groups compatible with having a single father (e.g. paternal half-siblings). In addition, Monte Carlo simulations were used to explore the pattern which would be produced if 1 to K fathers sired the offspring, with K representing the total number of offspring being assigned (e.g., the pattern produced if each offspring had a different father). The average r-values and standard deviations generated in the simulated scenarios were compared with the observed average r-values presented in the dendrogram. In order to evaluate how this method compares with direct parentage assignment, DADSHARE was run both with a dataset containing only those calves which were not assigned a putative father by the paternity assessment analysis as well as with a dataset including only those calves which were assigned putative fathers in the previous analyses.

A simulation-based approach, similar to that employed in previous parentage analyses conducted for mysticetes (Cerchio *et al.* 2005, Frasier *et al.* 2007), was used to evaluate how the observed patterns of

male reproductive success compared with those expected if all candidate males had an equal probability of fathering calves within a year (e.g., if mating were random). To make the results of the simulation comparable to those generated in the CERVUS analysis, simulations were based on the number of sampled candidate males included in each year of the analysis as well as the number of paternities which were assigned for those years. For each year of the analysis, candidate males were randomized, and then fathers were selected with replacement for the number of calves which were assigned paternity in that year. This process was repeated for each year of the study, and the number of calves fathered by each male was summed across years to generate the expected distribution of reproductive success for the study period under the expectation of random mating. This process was repeated 1000 times to generate the mean expected number of sampled males which were assigned paternity of zero, one, two, three, four or more calves under random mating.

To facilitate comparisons of male reproductive success with other species, the standardized variance (SV = variance/mean) in reproductive success was calculated as implemented in previous studies (e.g., Coltman *et al.* 1998, Frasier *et al.* 2007) and using both the results of the paternity analysis and results from the simulations based on random mating. This measure was based only on the reproductive success of males which were assigned as putative fathers of at least one calf during the study. The standardized variance in reproductive success is considered to be zero in truly monogamous species and to increase with the extent of polygyny (Boness *et al.* 1993).

Results

Genetic profiling

Summary statistics for the microsatellite loci used in the study are shown in *Table 1*. The total exclusionary probability of the multilocus genotypes used in the paternity analysis, as calculated by CERVUS when one parent is known, was high at 0.9999. The probability of identity (P_{ID}, Paetkau and Strobeck 1994) was estimated to be 2.83×10^{-13}, indicating that the loci utilized in the study provided high power to resolve relationships between individuals. The more conservative P_{ID-sib} (Evett and Weir 1998) was also calculated to account for the possible presence of related individuals within the dataset. This estimate was low (1.38×10^{-5}), suggesting that the data would be able to distinguish between any full siblings included as candidates. Rechecking of ~20% of all genotypes did not identify a substantial source of error.

Paternity assignment

When no mismatches were allowed between the genotype of candidate males and that of the mother-calf dyad (hereafter referred to as the "stringent criterion"), paternities were assigned for 26 (45.6%) of the 57 calves. All paternities were assigned at the 95% confidence level. One calf had a genotype which matched that of two candidate males; for the remaining 25 calves only one possible match was identified. No putative fathers were identified for the remaining 31 calves.

Incorporating an error rate of 0.01 (later referred to as the "relaxed" criterion), the CERVUS analysis supported all of the assignments previously made and identified putative fathers for an additional four calves, such that paternity was resolved for 52.6% of calves in the study. The four additional assignments included putative fathers with one (n=2) to two (n=2) mismatches with the genotypes of the mother-calf dyads. All CERVUS assignments were supported at the 95% confidence level, with the exception of the one calf whose genotype matched that of two candidate males with no errors, which was assigned at 80% confidence. Those calves which were not assigned putative fathers by the CERVUS analysis mismatched all potential candidates at ≥ 2 loci; for the majority (79%) of these calves, mismatches for ≥ 3 loci were present with all candidates.

The success of the paternity assignment varied greatly across the 12 years of the study (*Table 2*). Excluding years in which only one calf was sampled, the proportion of paternities assigned per year ranged from one year in which no paternities were assigned to years (n=3 using the relaxed criterion) in which putative fathers were assigned to two thirds of sampled calves.

The paternity assignment also varied greatly across the reproductive females included in the study (*Table 3*). Excluding females which only had one offspring during the study, the average proportion of calves with assigned fathers per female was 0.54 (relaxed criterion) and assignment success ranged from having no calves assigned putative fathers (n=3 females) to having all calves born during the study assigned putative fathers (n=5 females).

Male reproductive success

When no errors were allowed, 17 males were assigned paternity of the 26 calves, for an average of 1.5 (±0.72SD) calves per male (*Table 4*). Average reproductive success was slightly higher when calculated from analysis incorporating error, with 18 males assigned paternity for 30 calves and an average of 1.7 (±0.91SD) calves per male. In both cases, the majority of males (59% and 65% of assigned males for the stringent and relaxed analyses, respectively) were assigned paternity of only one calf each, although a small number of males were assigned paternity of 3-4 calves during the 12 seasons of the study.

These estimates of reproductive success do not incorporate males which were not assigned paternity of any calves. Although the number of candidate males varied across years of the study, 57-69% of candidate males were not assigned paternity of any calves over the twelve years of the study. Inclusion of these males would reduce estimates of average reproductive success to 0.47-0.71 calves per male.

As expected, none of the 13 males of known age (*i.e.*, first identified as calves, ranging from 5 to 11 years old during the season of fertilization) were identified as putative fathers during the study (*Table 5*). These males, as well as some proportion of the males of unknown age, were not sexually mature for all or part of the study. Of those males which were identified as putative fathers, all except one (only identified using the relaxed criterion) were identified prior to the 2000 season, and the majority (n=14, 77.8%) had been first identified by the end of the second season of the study (*Table 6*). All except two of the males were sighted at least once for six or more years of the study, with 14 males sighted for at least 8 of the 12 years of the study (*Table 6*). For the majority of assigned paternities (n=26 between 1999 and 2007), the putative father was identified on the feeding ground in the season prior to conception (77% of paternities) and/or the season following conception (73% of paternities).

Only one male was assigned paternity of 2 calves in any one season. Excluding this case, the average interval between successful reproductions was 2.8 years (relaxed criterion) to 3.75 years (stringent criterion). Although the maximum interval between assignments was six years, there were three males which were assigned as putative fathers early in the study and which were not assigned any additional calves for the following 8 to 11 seasons. Although some calves may have been lost before reaching the feeding ground, this finding suggested that intervals between successful mating may be even longer than illustrated with our dataset. The longest time span over which a male in the study was considered reproductively active was nine years, during which the male was assigned as the putative father of three calves.

In the seven to nine cases (stringent and relaxed criteria, respectively) in which a reproductive female had more than one calf assigned to a putative father, there was only one case in which the same father was assigned more than one calf of the same female. Female Q had three calves during the study, and all three calves were assigned to the same male (I) under the relaxed criterion. One of these calves mismatched the assigned father at two of the 13 loci and was only assigned to the male in the relaxed analysis. One of the other two assignments for calves of this female was the case where the genotype of the calf matched that of two different putative fathers with no errors. Although male I was assigned as the most likely father, relatedness analysis (data not shown) suggests that the two putative fathers may represent a parent-offspring pair. Given that this was the only case in which more than one putative father had a genotype which matched that of a calf at all loci, the paternity analysis seemed to generally perform well at discriminating between relatives. However, it is possible that in at least this case the candidate male with the highest likelihood of being the true father shared a different relationship to the calf.

DADSHARE estimated that 15 males were likely to account for the 27 calves not assigned fathers in the CERVUS analysis. Average reproductive success among these 15 males was 1.8 calves per male. Relative

to the paternity analyses, a larger proportion of these unsampled males (53%) were assigned paternity for two calves each during the study (*Graph 1*), suggesting these males may have greater reproductive success on average than those males which were sampled. When the DADSHARE analysis was run using only those calves which were assigned to putative fathers, 18 putative fathers were needed to account for the 30 calves, which corresponds exactly with the number of putative fathers identified in the "relaxed" CERVUS analysis. Similar to the results for the unassigned calves, however, the distribution of reproductive success among these 18 males was somewhat different than the observed pattern, with more males assigned as the putative father of two calves during the study. Differences in reproductive success between the sampled and unsampled males may be an artifact of differences in the resolution of the two methods.

At least some of these unsampled males may be accounted for among the 28 animals which have been photographically identified on the Sakhalin feeding ground but which have yet to be genetically sampled. One of these is presumed to be a female based on its close and prolonged affiliation with a calf during the one season it was sighted, and nine are animals first identified as calves (≤ 9 years of age at the end of the study) and may not have been reproductively mature for much, if any, of the study period. This leaves at least 18 animals of unknown sex or age which have been identified on the feeding ground but are not represented in the sample set. Although some proportion of these animals may be males contributing to reproduction in the population, based on the overall sex ratio of the population (58% male, Weller *et al.* 2008a), it is unlikely that all of the missing males are included in this group of animals. However, obtaining samples from these animals could potentially increase the success of the paternity assessment.

When the observed results were compared with those generated via the simulation of random mating (*Graph 2, Graph 3*), the average number of calves per father was significantly higher in both the relaxed ($p \leq 0.014$) and the stringent ($p \leq 0.037$) analysis. These results were due to significantly fewer than expected candidate males which were assigned only one offspring in the analysis ($p \leq 0.036$, stringent criterion; $p \leq 0.039$, relaxed criterion). The average numbers of males assigned paternity of two calves were similar between the simulated and observed results, while the average number of males assigned three calves over the study period was higher in the observed than the simulated results, although the differences were not significant. In addition, the number of males which were not assigned the paternity of any calves during the study was significantly higher than would be expected under random mating for both the stringent- and relaxed- criterion analyses ($p \leq 0.037$ and $p \leq 0.007$, respectively).

The standardized variance calculated from the results of the paternity analysis (SV_{obs}=0.42) was higher than that calculated from the data simulated under expectations of random mating (SV_{exp}=0.27). When compared to other mysticete studies, the SV_{obs} was most similar to the value calculated in the study of paternity in North Atlantic right whales (SV_{RW}=0.35; Frasier *et al.* 2007) and was higher than that calculated for the humpback whale population (SV_{HW}= 0.23; Cerchio *et al.* 2005).

DISCUSSION

Paternity assessment

Approximately half (46-53%) of the calves sampled on the Sakhalin feeding ground were assigned putative fathers which had been identified and sampled in the same area. Using the stringent criterion, all assignments were supported at the 95% confidence level, and the panel of loci utilized in this study provided sufficient resolution to discriminate between all possible candidates using a simple exclusion approach in all except for one case. Even when the more relaxed criterion was applied, all except one of the assignments was supported with high confidence (95%), and only four additional paternities were assigned. Assignment success was in relatively close agreement for both criteria, and it is likely that the true patterns of paternity are encompassed within this range of estimates.

Similar paternity assignment success rates have been generated in other studies of mysticete mating systems, including those conducted for humpback whales in the Mexican Pacific (32.5 to 49.6 %, Cerchio *et al.* 2005) and for North Atlantic right whales (41.4 to 62.1%, Frasier *et al.* 2007). Although sampling in the humpback whale population was not as complete, it is notable that in both our study and the North

Atlantic right whale study, in which an estimated 74% of animals which were considered alive had been sampled, a very high proportion of photographically identified individuals had been sampled. As such, the success rates of the paternity assignments were somewhat less than might be expected given the overall pattern of sampling. As discussed below, this pattern may have implications for our understanding of the population's status.

Distribution of male reproductive success

A mild skew in male reproductive success was detected over the 12 seasons covered by the study. Although most (56-59%) of the putative fathers identified were assigned paternity of only one calf each, this proportion was lower than that predicted in the simulations based on random mating. While not statistically significant, more males than expected were assigned paternity of three to four calves during the study, suggesting that some males achieved higher reproductive success than others. These results are consistent with those observed in both humpback whales and right whales, both of which demonstrated mild skews compared to random mating expectations (Frasier *et al.* 2007, Cerchio *et al.* 2005). However, in all cases the skew was slight, with most males siring only one calf during each of these studies. While the differences in reproductive success among males were relatively small over the course of the study, continuation of this pattern over the lifespan of these individual males would result in a substantially higher reproductive advantage for some males. However, if male reproductive success varied with age, the advantage gained by individuals during the study period would level out over time.

Based on testes to body size ratios, both gray and right whales are thought to utilize sperm competition (Brownell and Ralls 1986). The standardized variance of reproductive success, a measure often used for comparisons across species, was found to be high in North Atlantic right whales relative to values found in other aquatically mating species (Frasier *et al.* 2007), including a population of humpback whales (Cerchio *et al.* 2005). These results suggested that sperm competition may result in higher variance in reproductive success when compared to tactics employed by some other marine mammals (Frasier *et al.* 2007). The standardized variance estimated for western gray whales was comparable to that found in North Atlantic right whales, providing further support for the role of sperm competition in generating variance in reproductive success among males.

A high proportion of sampled males were not assigned any offspring during the 12 seasons of the study. These findings may suggest that many of the animals of unknown age were too young to successfully compete for mating opportunities. Rice and Wolman (1971) found that 24% of the animals from their sample were sexually immature and estimated that the total proportion of immature animals in the eastern gray whale population was approximately 44-61%. If the results of the paternity analysis are combined with the results of the DADSHARE analysis, the proportion of males (53-54%) that are potentially too young to reproduce falls within the range estimated by Rice and Wolman (1971). It is important to note, however, that successful fertilization not only necessitates that males are sexually mature but also that they are able to successfully compete for fertilization opportunities. Therefore, estimates derived from the paternity analysis are not necessarily representative of the proportion of animals which have not reached sexual maturity. In addition, this estimate assumes that the unsampled males are only those which are contributing to reproduction; if some proportion of non-breeding males has also not been sampled off Sakhalin, this percentage would be lower.

The results suggest a lack of mate fidelity among breeding pairs, with only one female with more than one calf assigned to the same male. These findings agree with expectations based on morphology and behavior. Similar results have been found in paternity analyses in humpback whales (Clapham and Palsboll 1997) and right whales (Frasier *et al.* 2007).

Identification of reproductive males

The paternity assignment identified 17 to 18 males as putative fathers, and analysis of relatedness patterns among the calves with unassigned paternities suggested that approximately 15 additional reproductive males have yet to be sampled. Twenty-four females were determined to be the mother of at least one calf in the western population between 1995 and 2007 (Weller *et al.* 2008a). Combining this information suggests

that approximately 57 animals are capable of reproduction. These numbers are slightly higher than previous estimates (Weller *et al.* 2002), which indicated that the number of mature individuals was approximately 39-49 animals if the population was growing and 55 animals if the population was stable. These estimates were based on parameters (*e.g*., percent of immature animals) derived from the eastern gray whale population and on the western population's size in 1999. Integrating information on the number of putative fathers with the number of females known to reproduce provides a more direct assessment of the number of animals contributing to reproduction in the population, including those which may not be regularly sighted on the primary feeding ground. Although slightly higher than previous estimates of the number of mature animals, the estimate incorporating the results of the paternity analysis supports the need for continued concern over the small size of the population. In particular, the low number of reproductive females may limit the population's growth and recovery (Weller *et al.* 2008a).

Although one of the putative fathers was not identified until the last year of the study, all other males identified as putative fathers were identified early in the study. These animals demonstrated a high degree of seasonal site fidelity to the primary feeding area, indicating that at least this subset of reproductive males are regular visitors to the Sakhalin area. Although some of the unsampled males may be represented among those animals which have been sighted on the feeding ground but not yet sampled, it seems likely that at least some of the "missing fathers" are animals which do not utilize the Sakhalin feeding ground on a regular basis.

The majority of the putative fathers had four of the five most common haplotypes found in the western population. Only two (one of which was identified only in the relaxed analysis) of the nine males which have haplotypes considered to be rare in the western population were identified as putative fathers. These "rare haplotype males" have been hypothesized to represent possible dispersers from the eastern population (Lang *et al*. 2010), although additional analyses evaluating this hypothesis have yet to be conducted. However, these results suggest that the majority of these "rare haplotype males" may not be currently contributing to reproduction in the population.

No males of known age were identified as putative fathers during the study; therefore, no conclusions about the minimum age at which males attain reproductive success can be derived from these results. Based on whales taken off central California under special permits between 1959 and 1969, the age of sexual maturity for both males and females was estimated to range between six and 12 years of age, with a median of nine years (Rice and Wolman 1971, Rice 1990). However, age at sexual maturity is not necessarily representative of age of first reproduction, particularly for males which may need to compete for successful fertilization. The lack of assigned fathers among the known-age males suggests that the age of first reproduction in males may be later than in females. Despite similar limitations in sample size, two females of known age (seven and eleven), out of 17 possible through the 2009 season, have been identified with calves (Bradford *et al.,* submitted). In right whales, paternity analysis suggested that most males do not attain their first successful mating until they were almost twice as old as the average age of fertilization for females (~15 years in males as compared to ~8 years for females; Frasier *et al.* 2007). Although no direct evidence was provided in this study, the lack of paternities assigned to males of known age ($\leq$ 11 yrs) suggests that a similar pattern may be true in western gray whales.

Conservation implications

Despite the high proportion of sampled individuals, the paternity analysis was only able to identify putative fathers for about half of the animals first sighted as calves on the Sakhalin feeding ground. Some of the "missing fathers" may be accounted for by the animals which have been sighted off Sakhalin but not genetically sampled. However, these results suggest that many of the males which are contributing to reproduction in the population may not be regular visitors to the Sakhalin feeding ground, raising questions about the identity and habitat use patterns of these individuals.

A potential explanation for the high proportion of unassigned paternities in the western gray whale population is that some gene flow with the eastern gray whale population may be occurring. Previous work has demonstrated that the eastern and western populations are genetically distinct (LeDuc *et al.* 2002, Lang *et al*. 2010); however, the low level of nuclear differentiation identified between the two populations raised

the possibility that some limited degree of interchange may occur (Lang *et al*. 2010). Such interchange might be characterized by mixing of animals from the two populations on the feeding ground, or might involve some degree of interbreeding. If gene flow between eastern and western populations occurs on a regular basis, then the percentage of candidate fathers that have been genetically sampled would be greatly reduced and a lower assignment success would be expected. Running the paternity analysis with the eastern males included did not identify any additional paternities (see Supplementary Information), but given the very small percentage (<1%) of the eastern population that has been sampled, this result is not very meaningful. However, although genetic drift acts strongly to maintain distinctiveness in small populations, the degree of interbreeding needed to account for the unassigned paternities (~50% of the reproduction in the population) would be likely to dissolve differentiation between the two populations.

If we assume that mating occurs primarily while on migratory routes as has been described in eastern gray whales, two other considerations are important. First of all, if any of the males identified off Sakhalin are animals which originated from the eastern Pacific, then they have a high probability of already being sampled. Therefore, interpopulation breeding between animals of eastern origin and females sampled off Sakhalin can only be used as an explanation for the "missing fathers" if the eastern males demonstrate lower levels of fidelity to the Sakhalin feeding ground and/or utilize other feeding areas but migrate along routes commonly used by Sakhalin animals. Secondly, interbreeding with the eastern population could occur if reproductive females which utilize the Sakhalin feeding area then return to the eastern Pacific to overwinter. Given the increased energetic demands of pregnancy and lactation, females are generally considered to be less likely candidates for dispersal than are males. In addition, of the 18 females which had multiple calves during the study period, the majority (83%, n=15) had at least one calf which was assigned a putative father among the animals sampled off Sakhalin, linking these breeding pairs to the use of common migratory routes during at least some seasons of the study.

An alternate explanation for the unassigned paternities, however, is that many of the males which are contributing to reproduction in the western population utilize other areas in the western Pacific to feed and are rarely found in the waters off Sakhalin. Similar reasoning was invoked to explain the relatively low paternity assignment success observed in North Atlantic right whales, in which only 51% of fathers were identified despite presumed high rates of sampling (69% of identified males; Fraser *et al.* 2007). The discrepancy between the number of assigned paternities and the proportion of whales which were thought to be sampled led the authors to conclude that the size of the North Atlantic right whale population is slightly larger than previously estimated. In addition, information from paternity analysis, in combination with photo-identification records suggesting that as many as one-third of the identified animals were "missing" during a given season (*i.e*., could not be accounted for within areas known to be utilized by this species), supported the existence of additional habitat(s) utilized by North Atlantic right whales but not yet located by researchers.

The results of the paternity analysis in the western gray whale population may suggest a similar pattern. Although it is possible to account for the "missing fathers" among animals identified but not sampled while on the Sakhalin feeding ground, it is unlikely that such a high proportion of the unsampled animals are reproductive males. However, sightings of animals identified as western gray whales have been made in other areas of the Okhotsk Sea (Weller *et al.* 2002), as well as the southwestern Bering Sea (Weller *et al.* 2003) and southeastern Kamchatka (Tyurneva *et al.* 2009). In addition, a relatively high proportion (n=39 of 78; 50%) of the whales sighted off southeastern Kamchatka have not been sighted on the Sakhalin feeding ground (Tyurneva *et al.* 2009). Although these individuals may be of eastern origin, they may also represent western gray whales which use the Sakhalin area infrequently or not at all. These observations suggest that at least some animals in the western population may range more widely during summer and may not have been identified on the Sakhalin feeding ground.

Patterns of relatedness among the unassigned calves suggest that the "missing fathers" may number approximately 15 different animals. Current population assessment models, which indicate that the population contains approximately 130 animals, assume that all western gray whales are sighted off Sakhalin, although not necessarily in all seasons (Cooke *et al.* 2008). The results of the paternity analysis suggest that this assumption may be violated, although the relatively small number of "missing fathers", some of which may be accounted for by photographically identified but not sampled individuals, likely

wouldn't change estimates of the population's size or trajectory dramatically. However, these results raise questions about the proportion of animals of other classes which may also not be accounted for in current estimates. Although little is known about the sex of most animals sighted in other parts of the Okhotsk Sea and eastern Kamchatka, sightings of mother-calf pairs between 1995 and 2008 have been largely confined to the waters of the primary feeding ground off Sakhalin, with only one sighting of a female with a calf in other parts of the range (Tyurneva *et al.* 2009). This female had previously been identified with a calf while utilizing the Sakhalin feeding ground. Thus, although females may range more widely during some seasons, they appear to exhibit particularly strong fidelity to the primary feeding ground in years when they have produced a calf. As such, the proportion of sampled to unsampled reproductive females is likely to be higher than the ratio suggested for males. Interestingly, a male bias has already been documented to exist among those western gray whales which have been sampled, such that approximately 60% of animals first identified (and sampled) as adults or subadults on the Sakhalin feeding ground are males. Results of the paternity analysis suggest that this male bias could be more pronounced than previously estimated.

Conclusions

Much of our understanding of the role that males play in the mating system of baleen whales is based on general patterns derived from behavioral and physiological data as well as predictions based on known differences in the reproductive cycles of males and females. While genetic analyses can provide valuable information on the distribution of reproductive success among males, in many species such studies are limited by the difficulty of collecting a sample set that is representative of population patterns. Due in large part to its small size and the high degree of site fidelity demonstrated by individuals, the western gray whale population is one of the most thoroughly sampled of all mysticete populations, providing a rare opportunity to learn more about the mating system of baleen whales. The findings presented here indicate that the distribution of reproductive success in this small population is similar to that described in North Atlantic right whales. Such comparisons suggest that sperm competition may create differential reproductive success among males, but that the degree of skew is mild in comparison to terrestrially mating mammals. Longer-term studies are needed, however, to determine how differential reproductive success over the relatively short time span of this study compares with patterns produced over the lifespan of individuals.

The lower-than-expected success rate in the paternity assignment for western gray whales raises many questions and suggests that the structure of this population may be more complicated than previously thought. In particular, the results presented here suggest that some animals which are part of the western population may not routinely visit the Sakhalin feeding area. Although this group of "missing fathers" may be small in number, they play a significant role in the reproduction of the population. As such, increasing our understanding of the habitat use and behavior of these animals is important. Genetic sampling of animals identified in other areas of the western Pacific, particularly those which have been sighted off Kamchatka but have not been identified as animals utilizing the Sakhalin feeding area, is one avenue that may elucidate habitat use of animals outside of the primary feeding area. Genetic analysis could be used to determine if these animals are any of the "missing fathers", and genetic assignment tests could be used to better understand if these animals originated from the eastern or western populations.

Although the relatively high proportion of calves which could not be assigned fathers is puzzling, assignment of putative fathers for approximately 50% of sampled calves provides strong evidence for intrapopulation breeding among animals demonstrating fidelity to the western feeding ground. Most females had at least one calf which was assigned a putative father from among the animals sampled off Sakhalin, suggesting the use of common migratory routes among these animals and the putative fathers which were identified. In the future, combining the results of the paternity analysis with genetic assignment tests may provide additional information on the extent and nature of any dispersal which may be occurring between the eastern and western populations.

ACKNOWLEDGEMENTS:

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Table 1. Diversity of the microsatellite loci utilized in the parentage analysis as calculated in CERVUS. The number of alleles (k), observed (H_{obs}) and expected (H_{exp}) heterozygosities, and polymorphic information content for each locus are shown. No loci were found to be out of Hardy-Weinberg equilibrium. The probability for non-exclusion of a parent pair, the probability of identity (assuming Hardy-Weinberg equilibrium), and the probability of identity assuming full siblings are represented in the data area are also shown.

Locus	k	H_{Obs}	H_{Exp}	PIC
D17t	14	0.846	0.896	0.881
EV14t	8	0.701	0.752	0.71
EV37	16	0.923	0.856	0.835
EV94t	9	0.782	0.754	0.708
Gata028	5	0.795	0.751	0.702
Gata098	6	0.615	0.604	0.562
Gata417	7	0.688	0.641	0.569
Gt023	7	0.654	0.685	0.632
RW31	9	0.859	0.832	0.805
RW48	5	0.39	0.414	0.386
SW10t	9	0.808	0.783	0.748
SW13t	8	0.649	0.648	0.58
SW19t	7	0.744	0.648	0.595
Overall	8.5	0.73	0.71	0.67

Non-exclusion probability (parent pair):	7.00×10^{-8}
Probability of identity:	2.83×10^{-13}
Probability of identity (sibling):	1.38×10^{-5}

Table 2. Paternities assigned for western gray whale calves in each season of the study, including the year in which the calves were born, the number of sampled males included as candidates for each year, the number of sampled mother-calf (M-C) pairs, and the number and percentages of paternities assigned under the stringent and relaxed criteria. Unless otherwise noted, all paternities were assigned at 95% confidence.

Year	No. of sampled males	No. of sampled M-C pairs	Paternities Assigned			
			Stringent		Relaxed	
			No. Assigned	%	No. Assigned	%
1995	42	3	1	33	1	33
1997	42	1	1	100	1	100
1998	42	5	1	20	2	40
1999	42	1	1	100	1	100
2000	42	2	0	0	0	0
2001	44	6	3	50	3†	50
2002	44	6	3	50	4	67
2003	46	10	5	50	5	50
2004	49	6	4	67	4	67
2005	50	5	1	20	2	40
2006	53	3	1	33	1	33
2007	55	9	5	56	6	67
Total	55	57	26	46	30	53

†One paternity resolved at 80% confidence

Table 3. Paternities assigned for calves of each reproductive female, including the number of sampled offspring for each reproductive female, and the number and percentage of offspring which were assigned a putative father in the analysis under both the stringent and relaxed criteria. Unless otherwise noted, all paternities were assigned at 95% confidence.

Mother's ID	No. of sampled offspring	Stringent No. Assigned	%	Relaxed No. Assigned	%
A	1	0	0	0	0
B	4	1	25	2	50
C	3	1	33	1	33
D	4	3	75	3	75
E	2	1	50	2	100
F	3	0	0	0	0
G	5	1	20	1	20
H	5	3	60	3	60
I	2	2	100	2	100
J	2	2	100	2	100
K	1	0	0	0	0
L	2	0	0	0	0
M	2	1	50	1	50
N	4	2	50	2	50
O	1	1	100	1	100
P	2	1	50	1	50
Q	3	2	67	3	100
R	3	1	33	1	33
S	3	3	100	3	100
T	2	0	0	0	0
U	2	1	50	1	50
V	1	0	0	1	100
Total	57	26	0	30	0.53

†One paternity resolved at 80% confidence

Table 4. Distribution of reproductive success among the putative fathers identified in the paternity analysis. Included are the candidate father's ID, his mtDNA haplotype, the year in which he was first photographically identified, and the number and birth year of the offspring he was assigned under the stringent and relaxed criteria analyses. Mean reproductive success for all fathers, along with the standard deviation, is shown at the bottom of the table.

| Father ID	Year Identified	Father's Haplotype	Stringent	Relaxed	
			No. of Offspring	No. of Offspring	Year(s)
A	1994	A	2	2	2002, 2004
B	1995	B	1	1	2004
C	1997	A	1	1	2007
D	1995	D	1	1	1999
E	1997	A	3	3	1997, 2002, 2006
F	1995	B	1	1	2007
G	1997	B	1	1	2003
H	1997	D	1	1	2003
I	1995	A	2	4	1998, 2001, 2002, 2003
J	1995	C	1	1	2003
K	1997	A	3	3	2002 & 2007 (2)
L	1998	J	1	1	1998
M	1995	B	1	1	1995
N	1994	D	2	2	2004 & 2005
O	1999	B	2	2	2001 & 2003
P	1999	B	2	2	2001 & 2007
Q	1995	A	1	2	2004, 2005
R	2007	Q		1	2007
Average:			1.5	1.7	
SD:			0.72	0.9	

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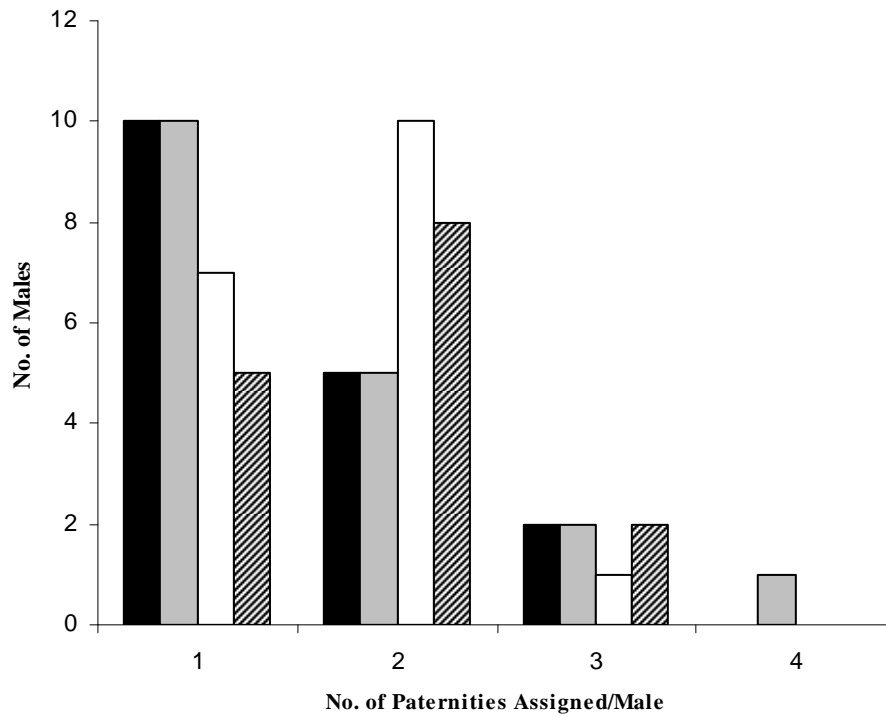
Graph 1. Comparison of the number of males which were assigned one, two, three, and four offspring in the stringent CERVUS analysis (shown in black), the relaxed CERVUS analysis (shown in gray), and the DADSHARE analysis using only those calves which were assigned putative fathers in the relaxed analysis (shown in white), and the DADSHARE analysis using only those calves which were not assigned putative fathers in the relaxed analysis (black and white pattern).

Table 5. Number of known age males for each year of the study. Included are the year of each study, the number of mother calf (M-C) pairs sampled in each year, the number of paternities assigned, the number of males in each age category (5 to 11) for each year, and the total number of known age males included in the paternity analysis for each year. Age categories refer to the approximate age of the male in the season during which fertilization would have occurred.

Year	No. of M-C pairs	No. of assigned paternities	Approximate age of males during reproduction (Year -1)							No. of known age males
			5	6	7	8	9	10	11	
2001	6	3	2							2
2002	6	3		2						2
2003	10	5	2		2					4
2004	6	4	3	2		2				7
2005	5	1	1	3	2		2			8
2006	3	1	3	1	3	2		2		11
2007	9	5	2	3	1	3	2		2	13

Table 6 Sighting patterns of males identified as putative fathers in the paternity analysis. Included are the father's ID number and the date on which he was first photographically identified. Years in which the male was sighted at least one are shaded in gray; numbers in each cell refer to the number of calves identified in each season which were assigned to that father. The * symbol is used to denote offspring only assigned under the relaxed criteria. The percentage of years that each candidate male was sighted on the Sakhalin feeding ground of all years of the study following his initial identification is also shown.

ID	1995	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	% of years sighted
A							1		1				100%
B									1				92%
C												1	82%
D				1									67%
E		1					1			1			100%
F												1	83%
G								1					73%
H								1					91%
I			1			1*	1*	1					100%
J								1					83%
K							1					2	73%
L			1										80%
M	1												75%
N									1	1			69%
O						1		1					44%
P						1						1	67%
Q									1	1*			58%
R												1*	100%
Total	1	1	2	1		3	4	5	4	2	1	6	30
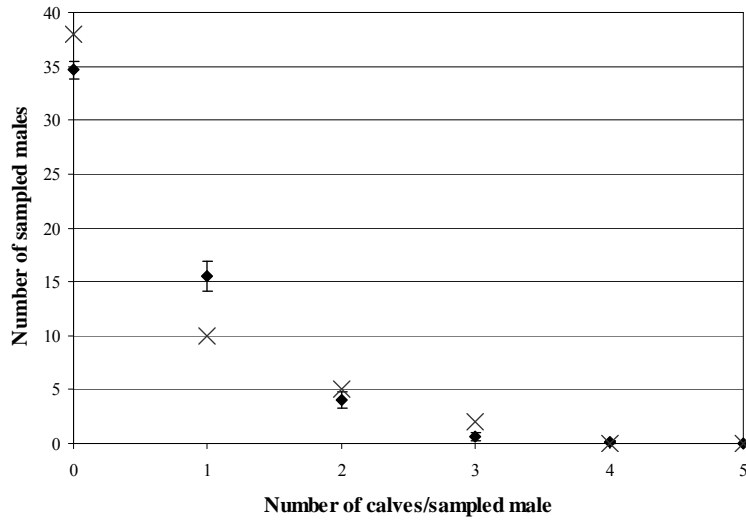
Graph 2. Graph comparing the expected distribution of paternities based on simulations of random mating with the observed results for the stringent criterion analysis. Expected values, with error bars representing standard deviations, are shown with black diamonds, while the observed values are displayed with an X.
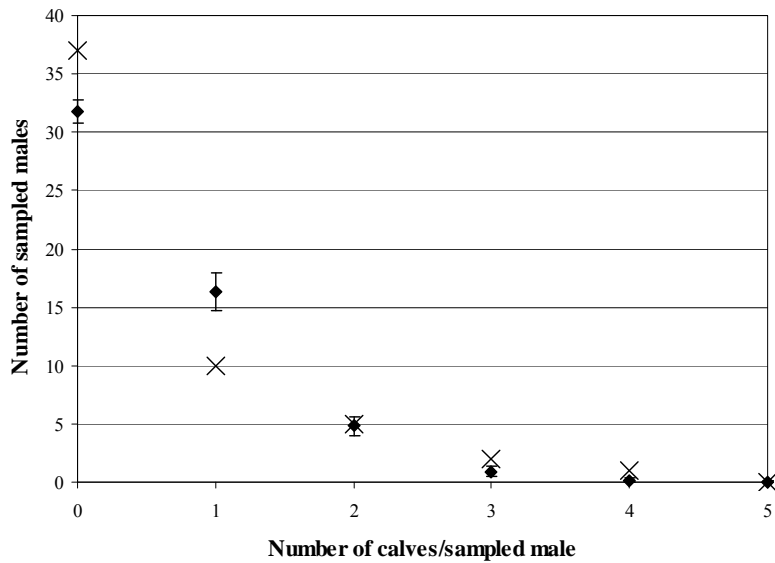
Graph 3. Graph comparing the expected distribution of paternities based on simulations of random mating with the observed results for the relaxed criterion analysis. Expected values, with error bars representing standard deviations, are shown with black diamonds, while the observed values are displayed with an X.