BRIEF REPORT

Testing for a Historical Population Bottleneck in Wild Verreaux's Sifaka (*Propithecus verreauxi verreauxi*) Using Microsatellite Data

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The degree to which historical human activities negatively impacted past and present lemur species is a long-standing question in primatology. At present, most evidence addressing this issue comes from archaeology, paleontology, and behavioral studies. Genetic data provide another source of evidence. In this study, six microsatellite loci, genotyped on more than 360 wild Verreaux's sifaka, are used in order to test the hypothesis that this population experienced a population bottleneck in the last 2000 years. Excess heterozygosity is compared with the heterozygosity expected under mutation-drift equilibrium in order to test for the genetic signature of a rapid population contraction in the past. The results indicate that the sifaka population did not experience a population bottleneck. Various methodological and conceptual implications of this result are discussed. Am. J. Primatol. 70:990–994, 2008. © 2008 Wiley-Liss, Inc.

INTRODUCTION

Like all human–animal relationships, the relationship of lemurs to the human inhabitants of Madagascar is complex, varies by region and circumstance, and cannot be characterized by a single cultural attitude. In some regions of Madagascar it is currently "fady" or taboo to hunt and kill lemurs [Loudon et al., 2006], whereas in other regions particular species are portents of impending sickness and killing them is allowed [e.g., Simons & Meyers, 2001]. Despite the current regional differences regarding lemur livelihood, one topic that has gathered a lot of attention is the degree to which the arrival of humans to Madagascar negatively impacted past and present lemur species. Similar to the present day, most researchers suggest that the human impact on lemur populations varied from region to region [e.g., Dewar, 2003]. This is because the evidence suggesting a connection between human activities and lemur population disruption is often indirect. The basic facts regarding this topic are the following: (1) humans arrived to Madagascar about 1000-2000 years ago; and (2) during this same time period a lot of lemur species (now recovered as subfossils) disappeared, as evidenced from changes in faunal assemblages, paleofungal analysis, and archaeological evidence [Burney et al., 2004; Dewar, 2003]. Hypotheses put forth to explain the lemur extinctions either implicate single factors (e.g., targeted hunting, climate change, fire-driven landscape changes, disease, invasive herbivorous species) or suggest a combination of factors. These hypotheses are most recently evaluated in Dewar [2003], Burney et al. [2004], and Godfrey and Irwin [2007].

At present all the evidence hypothesizing a connection between human activity and lemur extinctions or population disruption comes from archaeology, paleoecology, comparisons of extant lemur behavioral ecology and life history patterns with other primates, and, of course, the recovery of subfossil lemur taxa that are no longer around today [e.g., Burney, 1999; Burney et al., 2003; Dewar, 1984; Godfrey & Irwin, 2007; Godfrey et al., 2006; MacPhee & Burney, 1991; van Schaik & Kappeler, 1996]. There is, however, another body of evidence that can speak to this issue. This evidence comes from genetics. The signature of past demographic events, such as population growth or population bottlenecks, are registered in the genotypes and allele frequencies of present day populations [Cornuet & Luikart, 1996; Luikart & Cornuet, 1998;

Luikart et al., 1998]. Although genetic data have long been used to make inferences about past demographic events in human history, few studies have applied these genetic techniques to wild primate populations in order to test paleodemographic hypotheses [see Goosens et al., 2006; Storz et al., 2002]. In this study, I use microsatellite loci in order to test the hypothesis that a wild population of Verreaux's sifaka (*Propithecus verreauxi verreauxi*) has experienced a population bottleneck in the last 2000 years. This population resides at Beza Mahafaly Special Reserve (BMSR) in southwest Madagascar.

MATERIALS AND METHODS

The basic technique for determining a recent population bottleneck is straightforward. A population bottleneck has different effects on heterozygosity and allelic diversity (i.e., the number of alleles at a locus). It is widely known that, all else being equal, heterozygosity is lost at a rate of $1/2N_e$ per generation (where N_e is the effective population size). As Allendorf and Luikart [2006] point out, if a population is reduced to two individuals during a bottleneck $(N_e = 2)$, it is expected to lose $1/2N_e = 1/2*2 = 0.25$ of its heterozygosity or, more simply, it is expected to retain 75% of its heterozygosity. These two individuals, however, can only have a maximum of four different alleles at a particular locus. Thus, allelic diversity is disproportionately influenced by a population bottleneck, since it is possible that before the bottleneck the locus in question had, for example, 20 alleles (which would constitute an 80% reduction in allelic diversity). The probability of losing an allele during a bottleneck is particularly high for alleles at low frequency (i.e., less than 0.01), but heterozygosity is still lost at a constant rate: $1/2N_e$ [Allendorf & Luikart, 2006].

Population bottlenecks have the effect of temporarily knocking a population out of mutation-drift equilibrium (i.e., where the addition of new alleles via mutation is balanced by the loss of alleles via drift) [Luikart & Cornuet, 1998]. It is possible to calculate the expected heterozygosity under mutation-drift equilibrium (Het$_{EQ}$) using simulations and a particular model of how mutations are introduced into the population. Two widely used mutation models are the infinite allele model (IAM) and the stepwise mutation model (SMM). These mutation models represent the two extremes of how new alleles are introduced into the population. The IAM considers any point mutation along a stretch of DNA within a locus to constitute a new allele, whereas the SMM counts new alleles along a stretch of DNA with respect to the addition or subtraction of particular subsets of DNA motifs. An additional mutation model, the two-phase model (TPM), has been proposed as an ''intermediate'' model that provides

a more realistic picture of how some DNA sequences evolve [Di Rienzo et al., 1994].

The key test for determining if a bottleneck recently occurred in a population is to examine the observed heterozygosity relative to Het$_{EQ}$ calculated from the allelic diversity in the sample. As discussed above, a population bottleneck will have a disproportionate effect on allelic diversity but less of an effect on heterozygosity. A bottleneck creates an excess level of heterozygosity in the population above what would be expected under Het$_{EQ}$. Over time, however, the population reaches a new equilibrium and the transient excess heterozygosity decreases. Thus, the ''window'' for determining a recent population bottleneck is transient and it is possible to only test for population bottlenecks that have occurred in the last $4*N_e$ generations ago, where N_e is the effective population size.

To test for a recent population bottleneck, I use six unlinked microsatellite loci, all in Hardy–Weinberg equilibrium, that were isolated from the sifaka population [Lawler et al., 2001]. Four hundred and twenty-six animals from 60 social groups were used in the analysis. Each locus was amplified on a minimum of 368 animals, as some loci were not amplified on every animal in the sample [the final sample size for each locus is more than sufficient to estimate heterozygosity and allelic diversity; Cornuet & Luikart, 1996]. Previous analysis suggests that the effective population size for this population is around 100 [Lawler, in press]. Sifaka females have an *average* age at first birth around 9 years (note, this is not the minimum observed age at first reproduction), suggesting that generation time is around 9 years [Lawler, in press]. Hence, if $N_e = 100$ and generation time = 9 years, $4*N_e*$generation time $= 4*100*9 = 3600$ years, which falls within the ''window'' of testing for a historical population bottleneck. Tissue samples for the genetic analysis were collected annually between 1988 and 1999, with permission granted by the government of Madagascar; the genetic analysis was conducted between 1999 and 2002. All necessary permits (CITES, IACUC, and biohazard) were obtained and approved before conducting this research.

I used the program BOTTLENECK to test for a recent population bottleneck [Piry et al., 1999]. This program generates the expected Het$_{EQ}$ from the number of alleles at a locus and the sample size using the SMM, TPM, and IAM mutation models; the Het$_{EQ}$ values are then averaged across loci and compared with the observed level of heterozygosity. The SMM and TPM are most appropriate for microsatellite data [Luikart & Cornuet, 1998], with the TPM providing a more realistic picture of mutational events in microsatellite loci [Di Rienzo et al., 1994; Piry et al., 1999]. Het$_{EQ}$ was calculated using the SMM and the TPM, the latter allowing

80% of the mutations to follow a two-phase mutation pattern rather than a strict stepwise mutation pattern. The program returns several nonparametric tests of whether heterozygosity deviates from that expected under Het_{EQ}. The most powerful of these tests—and the one employed here—is the Wilcoxon test. This test is particularly appropriate when less than 20 loci are used [Piry et al., 1999]. I use two different sets of individuals in my analysis: females and females+males. Verreaux's sifaka females at BMSR are generally philopatric [Lawler et al., 2003]; the use of females only is to control for the possibility that male migration into the reserve can introduce new alleles at low frequencies.

RESULTS

Under the SMM, the Wilcoxon test revealed no significant patterns of excess heterozygosity for either females (Wilcoxon test: $P = 1.0$) or females+ males ($P = 1.0$). Similarly, under the TPM, the Wilcoxon test did not reveal a significant pattern of excess heterozygosity for either females (Wilcoxon test: $P = 0.66$) or females+males (Wilcoxon test: $P = 0.98$). Overall, the genetic data do not suggest that the sifaka population has recently experienced a historical population bottleneck. The SMM results suggest a deficiency in heterozygosity, indicating population expansion, whereas the TPM results are more equivocal and only suggest a deficiency in heterozygosity using the females+males data set (see below).

DISCUSSION

As is evident from the results, the hypothesis that a historical population bottleneck occurred in the sifaka population can be rejected. Despite these negative results, several methodological and conceptual points are worth mentioning. Methodologically, it is important to understand what factors can confound the analyses conducted above. The bottleneck test can be viewed as somewhat "coarse-grained" in that it uses summary statistics averaged across loci in order to detect a recent population bottleneck, and it is sensitive to the mutation model used in the analysis. Cornuet and Luikart [1996] have run power tests under a variety of conditions (e.g., varying the average heterozygosity across loci, number of loci, sample size, and the severity and elapsed time since the bottleneck). They show that statistical power increases when sampling more loci than when sampling more individuals and, overall, power is reduced when loci follow an SMM vs. the TPM or the IAM model. For the present analysis, the statistical power to detect a bottleneck is minimally about 0.40–0.70 under the SMM. The range is due to the fact that the severity and elapsed time since the bottleneck—if it occurred—is not known. The actual

statistical power is likely to be larger than these minimal values as the sample size is over 350 individuals and power increases with the addition of more individuals [Cornuet & Luikart, 1996]. That said, one explanation for the lack of any signal in the sifaka data is that the current analysis lacks the power to detect a bottleneck even if one occurred.

Another problem is that this method has trouble separating out the genetic signal of a population bottleneck from that of subsequent rapid population growth, as the latter signal can obliterate the former, as found by Bonhomme et al. [2008]. During rapid population expansion, new alleles are introduced into the population rapidly via mutation. However, these new alleles are at a low frequency creating an excess of allelic diversity above what would be predicted from a nongrowing population at muta-tion-drift equilibrium. This is because low-frequency alleles contribute much less to average heterozygos-ity than alleles at intermediate frequency. Hence, a transient reduction in heterozygosity (or concomi-tantly, a transient increase in homozygosity) is predicted from rapid population growth [Maruyama & Fuerst, 1984]. Rapid population expansion and/or lots of male migration into the reserve, even if preceded by a population bottleneck, could account for the observed deficiency in heterozygosity in the sifaka data.

Genetic substructure in the study population is another methodological problem that can also skew these analyses. When independent populations are lumped into a larger group this can mimic the signal of rapid population decline [Goosens et al., 2006]. The sifaka population is genetically substructured at the level of the social group [Lawler et al., 2003]; however, at the regional level, where the entire population is divided into three zones—east, center, and west—there is minimal genetic differentiation among these three regions, $F_{ST} \leq 0.019$, suggesting that population structure is not likely to influence the present analysis. More complicated models have been developed for determining demographic history from genetic data that can circumvent many of the limitations discussed above. These include Bayesian hierarchical models and "approximate Bayesian computation" methods. These models detect past population growth rates as well as the elapsed time since population expansion/decline using locus-by-locus data [Beaumont, 1999; Beaumont et al., 2002]. These methods have been successfully applied to wild primate populations [Bonhomme et al., 2008; Goosens et al., 2006; Storz et al., 2002]. The application of these methods to the BMSR sifaka population will be presented elsewhere.

On the conceptual side, currently, there is a protective "fady" (or taboo) among the Mahafaly peoples against hunting/killing sifaka and ring-tail lemurs in and around BMSR. As this sifaka popula-tion has been historically situated in Mahafaly lands,

it is possible that ancestral and extant sifaka have not been targets of hunting [Loudon et al., 2006]. However, it is not clear when the fady against hunting/killing sifaka originated. If the fady is relatively recent in origin, then it is possible that humans inhabiting this region before the instigation of the fady hunted sifaka and/or pursued subsistence practices that ultimately destroyed sifaka habitat. Regarding the latter, bones from Verreaux's sifaka recovered from the nearby site of Taolambiby show evidence of cut-marks and butchery, likely owing to hunting [Perez et al., 2005]. Although this latter evidence is fairly conclusive, it does not speak to the magnitude of sifaka hunting in this area. Although the data from Taolambiby show that humans hunted some individual sifaka fairly recently [Burney et al., 2004], the genetic data suggest that the impact was insufficient to produce a genetic signature indicative of rapid population decline.

Populations that experience reductions in census size might not experience similar reductions in genetic effective population size (N_e). This occurs if the N_e of the population has always been low relative to census size. The effective size of the sifaka population, depending on how it is calculated, is about 100 [Lawler, in press]. This number is about 2.5 times lower than the current census size. If N_e has always been low relative to census size, the sifaka population could experience fairly large reductions in census size from droughts [Richard et al., 2002] or perhaps hunting [Perez et al., 2005], but these events would not always reduce N_e, and hence would not register as the signature of excess heterozygosity in extant genotypes [Cornuet & Luikart, 1996; also see Garza & Williamson, 2001]. Similarly, some extinction hypotheses, such as climate change, suggest a slow rate of extinction [see Burney et al., 2004]. A slow and steady population reduction is not likely to cause a statistically significant signal of excess heterozygosity in the sifaka genotypes and thus could not be detected using the present analysis. The Bayesian methods mentioned above can likely resolve this issue.

Obviously, using a single lemur species from a single site is not the best way to assess the larger question of how extant lemur populations were impacted by human activities in Madagascar. Numerous genetic samples from numerous sites and species would provide a better picture of whether lemur genotypes contain the historical signature of a population bottleneck. Although the results of this study are limited in scale, it is clear that genetic data provide yet another route of inquiry into the historical relationship between humans and lemurs. Perhaps no other geographic region is more amenable to testing explicit historical hypotheses regarding human–nonhuman primate interactions than in Madagascar. Given the debates surrounding the degree to which humans directly or indirectly altered the life history and behavioral patterns of extant lemurs, more genetic-based studies concerning the historical demography of lemurs are surely warranted.

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