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The Ghost of Genetic Diversity Past: Historical DNA Analysis of the Greater Prairie Chicken

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ABSTRACT: Most, if not all, of the "classic," often-cited examples illustrating the genetic effects of a population bottleneck are open to alternative explanations due to the lack of adequate control populations, that is, low levels of genetic variability are often assumed to be the result of a past population bottleneck without having any prebottleneck measures. Here we provide the first clear case history where both prebottleneck and postbottleneck measures of genetic variability have been collected from a natural system. Analysis of DNA from museum specimens of the greater prairie chicken *Tympanuchus cupido* from central Illinois revealed the loss of specific alleles (known to have been present earlier in this century) following a demographic contraction. Lost alleles included common ones present in all other populations sampled and others unique to the Illinois population.

Keywords: Tympanuchus cupido, historical DNA analysis, bottleneck, genetic diversity, microsatellites.

Genetic theory predicts that a population that has undergone a significant demographic contraction will lose considerable genetic variation as a consequence of reduced population size (Wright 1969; Nei et al. 1975; Chakraborty and Nei 1977; Lacy 1987). On the basis of this prediction, current low levels of genetic variability have frequently been used to infer past population bottlenecks (e.g., Bonnell and Selander 1974; O'Brien et al. 1983; Ellegren et al. 1996), often ignoring alternative explanations such as environmentally selected differences. However, to date, no study has convincingly demonstrated the genetic effects of a demographic bottleneck in a natural population, that is, directly quantifying genetic diversity before and after a bottleneck. With the advent of the polymerase chain reaction (PCR; Saiki et al. 1985), genetic analysis of extinct species or, when sources are available, populations from different time periods have been possible (Hagelberg et al. 1989; Ellegren 1991; Cooper et al. 1996). Using museum specimens of the greater prairie chicken *Tympanuchus cupido,* we provide the first case history where the loss of genetic variation in a wild population can be directly linked to a population bottleneck by comparing levels of genetic variability prior to and following a demographic contraction.

The greater prairie chicken is a grassland/prairie species with limited dispersal and a lek mating system. During the last century, populations have become increasingly affected by the loss of natural habitats because of human activities. In the state of Illinois, greater prairie chicken populations, estimated to include millions of individuals in the 1860s, began to decline dramatically from an estimated 25,000 birds in 1933 to 2,000 in 1962, 500 in 1972, 76 in 1990, and to less than 50 in 1993 (Westemeier et al. 1991). Today, the Illinois population in Jasper County represents one of two wild populations of prairie chickens east of the Mississippi river. Other extant populations in Kansas, Minnesota, and Nebraska have, in contrast, remained comparatively large, with wide geographic distributions and numbers ranging from 4,000 to more than 100,000 birds (Bouzat et al. 1998).

Recent studies suggest that, as a result of its demographic contraction, Illinois prairie chickens have lost genetic diversity (Bouzat et al. 1998). Compared to populations in Kansas, Minnesota, and Nebraska, with no known bottleneck histories, the Illinois population had the lowest estimate of mean heterozygosity per locus and about two-thirds of the allelic diversity present in the other populations. A definitive test of the hypothesis that a demographic contraction is responsible for the lack of genetic diversity would, however, require evaluating the genetic variability of the Illinois population prior to its decline, that is, looking for "ghost" alleles that were lost during the population bottleneck.

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We present genetic information on six microsatellite loci from 10 *Tympanuchus cupido* specimens of the Illinois population collected during the 1930s and five specimens from the 1960s, when its population size was comparable to the sizes of the current populations in Kansas, Minnesota, and Nebraska. Allelic diversity present in the early Illinois population is compared with that of the current Illinois population as well as with current populations in Kansas, Minnesota, and Nebraska, which have no known bottleneck histories.

Methods and Material

DNA Sampling and Extraction Methods

The DNA samples from the early Illinois population were obtained from feather roots of museum specimens from the Illinois Natural History Survey (INHS) ornithological collection. Most museum specimens were collected from the same population in Jasper County, Illinois, for which we estimated current levels of genetic variability (Bouzat et al. 1998). Two birds were collected from neighboring Richland County. Exclusion of these two individuals did not alter the interpretation of our results; they were therefore included in our study. Use of birds from Jasper County's prebottleneck population eliminates the possibility of detecting alleles that resulted from geographic differentiation in other populations. Initially, feather roots were rinsed with absolute EtOH and dried in a 1.5 mL microfuge tube. The DNA was isolated by digesting samples with Proteinase K in 400 μL of DNA extraction buffer (400 mM NaCl, 10 mM Tris-HCl [pH 8.0] 2 mM Na_2EDTA, 1% SDS). After phenol-chloroform extraction, DNA samples were concentrated and desalted with a Centricon 30 Ultrapurification System (Amicon, Inc., Beverly, Mass.). All museum samples were subjected to at least two independent DNA extractions. The DNA samples were then diluted to 30 ng/μL for further PCR amplifications. Genomic DNAs of current populations from Kansas ($n = 37$), Minnesota ($n = 38$), and Nebraska ($n = 20$) were extracted from blood samples of birds captured between 1992 and 1994. The DNA samples from current Illinois prairie chickens ($n = 32$) were extracted from tissue samples of birds collected from incidental mortalities occurring between 1974 and 1993 (see Bouzat et al. [1998] for sampling and DNA extraction protocols of modern populations). Six polymorphic microsatellite loci were amplified by PCR using fluorescently labeled primers (Bouzat et al. 1998). Polymerase chain reactions were set up in 20 μL volumes, each containing about 30–60 ng of DNA template, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 μM concentrations of each dNTP, 0.25 μM of each primer and 0.5 U of Taq polymerase enzyme. Polymerase chain reactions were performed in an MJ Research thermocycler with a 3-min denaturation step at 94°C followed by 34 cycles of 30 s denaturation at 94°C, 30 s annealing at 46°–50°C, 30 s of extension at 72°C, and then a final 5-min extension step at 75°C. Some samples were subjected to a second amplification reaction using 3 μL of the first PCR product as a template. Microsatellite products were then electrophoresed in an ABI Model 373A automated DNA sequencer (Applied Biosystems, Foster City, Calif.; see fig. 1). Microsatellite primers used did not cross amplify DNA from any other species in our lab (humans, cattle, and rheas), eliminating any potential for contaminated product. In addition, the risk of contamination from modern prairie chicken DNA was minimized due to the

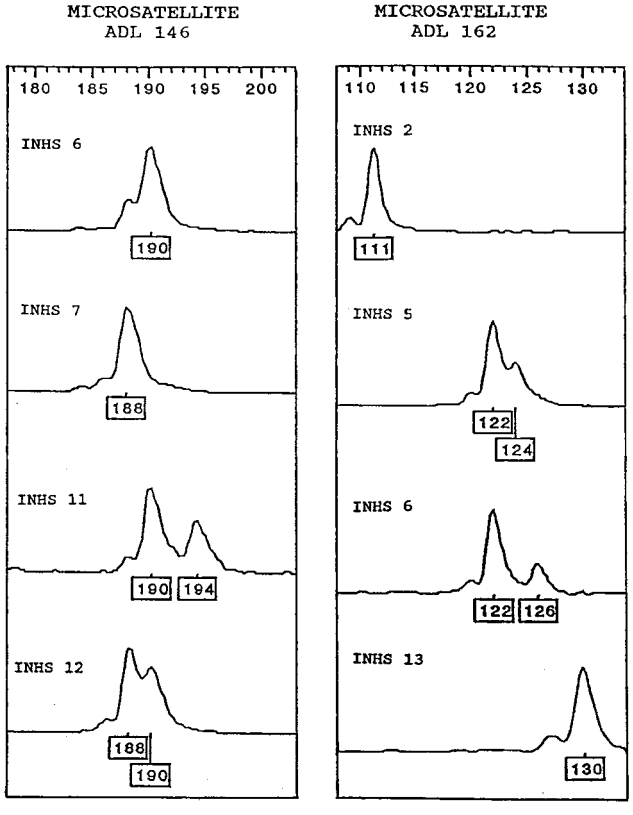

Figure 1: Example of electropherograms of microsatellites ADL146 and ADL162 in four Illinois Natural History Survey (*INHS*) museum specimens of the early Illinois population. Electropherograms were obtained by microsatellite DNA amplification and detection using an automated DNA sequencer. The values assigned to each peak indicate allele size in number of base pairs. Allele 194 of microsatellite ADL146 and alleles 124 and 126 of microsatellite ADL162 represent allelic variants found in the museum specimens that are shared by each of the other three populations (Kansas, Minnesota, and Nebraska) but that are currently extinct in Illinois. Allele 130 of microsatellite ADL162 was only present in the early Illinois specimens.

yields of DNA obtained from the museum specimens and the amplification of novel alleles not present in any of the modern populations (see "Results and Discussion"). Furthermore, all individual samples had no more than two alleles for any microsatellite locus, suggesting that samples were not cross contaminated with DNA from other individuals. Control and replicate reactions were also performed to ensure authenticity and repeatability of microsatellite products as well as to eliminate the possibility of having chimeric PCR products resulting from degraded DNA obtained from museum specimens. Polymerase chain reaction negative controls (without DNA) did not result in any amplification product. Amplification of DNA samples obtained from the same specimen by two independent extractions resulted in the same microsatellite product (repeated for DNA samples from up to seven individuals for a given microsatellite primer). Furthermore, when the same samples were subjected to independent PCRs, products of the same size and electropherograms of similar pattern resulted.

Statistical Analysis

Differences of allelic diversity per locus among the early Illinois birds and current populations from Illinois, Kansas, Minnesota, and Nebraska were tested by a two-way ANOVA with locus and population as main factors (Proc GLM, SAS Institute 1990). Interaction between locus and population was assessed by Tukey's nonadditivity test. Model assumptions were evaluated using residual analysis. Normality of residuals was assessed using normal probability plots and the Shapiro-Wilk test ($W = 0.96$; $P = .37$). Heteroscedasticity was evaluated by plotting log of residuals versus log of predicted values. Spearman correlation coefficients indicated that residuals had homogeneous variances ($\rho = 0.24$; $P = .20$). Model assumptions were not violated, thus justifying the use of an ANOVA to assess population and locus effects. To check for possible effects of different sample sizes of current populations, we plotted the log of residuals versus sample size per population and locus. Pairwise comparisons of the mean number of alleles per locus between populations were performed by the Student-Newman-Keuls test ($P < .05$; SAS Institute 1990). In addition, a randomization distribution to test for significant differences in the number of alleles among populations was also performed. Based on 5,000 random permutations of row values of the data matrix, we generated a distribution of the F statistics for the null hypothesis of no differences among populations. The significance level of the F statistic for testing population differences was calculated in two ways: one for F values assuming additivity (i.e., no interaction) and one for the F values where the error mean-square is adjusted for the Tukey form of interaction.

Results and Discussion

The specific alleles found in each of the present populations and in the early Illinois birds are shown in table 1. At each locus, alleles present in the current Illinois population represent a subset of the total alleles found in all other populations. In addition, Kansas, Minnesota, and Nebraska have few specific alleles. The fact that all alleles present in the current Illinois population are shared with other populations suggests that they were also present prior to the demographic contraction. In the museum specimens, we found 17 of the 22 alleles currently extant in Illinois (table 1). The five alleles missing probably reflect the rarity of these alleles (all but one have frequencies <0.07 in the current populations) and the small sample size of the early Illinois population. In addition to most of the currently extant alleles, we identified in museum specimens nine alleles not present in the current Illinois population (table 1). Five of these nine alleles were present in each of the other populations (Kansas, Minnesota, and Nebraska). The exceptions are one allele shared

Table 1: Individual alleles found at each microsatellite locus in each of the present populations (Illinois, Kansas, Minnesota, and Nebraska) and in the early Illinois birds

	Loci					
Population	*ADL42*	*ADL23*	*ADL44*	*ADL146*	*ADL162*	*ADL230*
Illinois	ABC	ABCD	A D F H	ABC	B E	C EFGHI
Kansas	ABCD	BCDEF	A CDEFGH	ABCDE	ABCDE	ABCDEFGHI
Minnesota	ABCD	ABCD	ABCDEFGH	ABC E	BCDE	BCDEFGHI
Nebraska	ABCD	ABCDE	ABCDEFGH	BC EF	BCDE	BCDEFGHIJK
Museum specimens	AB	BCD**E**	A D F	ABC **E**	B**CD***EFG*	**BCD**EFG I *L*

Note: Bold letters indicate alleles found in the museum specimens that have been lost as a result of its demographic contraction. Italic letters indicate alleles unique to the Illinois population prior to its demographic contraction.

Table 2: Number of alleles per locus found in each of the current populations of Illinois, Kansas, Minnesota, and Nebraska and estimated for the Illinois prebottleneck population

Locus	*Illinois*	*Kansas*	*Minnesota*	*Nebraska*	*Illinois prebottleneck**
ADL42	3	4	4	4	3
ADL23	4	5	4	5	5
ADL44	4	7	8	8	4
ADL146	3	5	4	4	4
ADL162	2	5	4	4	6
ADL230	6	9	8	10	9
Mean	3.67^{A}	5.83^{B}	5.33^{B}	5.83^{B}	5.12^{B}
SE	.56	.75	.84	1.05	.87
Sample size	32	37	38	20	15

Note: SE indicates standard errors of mean number of alleles per locus. Different letters indicate significant differences at $P < .05$ (see "Methods" for statistical analysis).

* Number of alleles in the Illinois prebottleneck population include both extant alleles that are shared with the other populations and alleles detected in the museum collection.

only with Kansas and Nebraska populations, and three alleles at microsatellite loci ADL162 and ADL230 that were unique to the Illinois population prior to its demographic contraction. As expected, of those alleles that were lost through the demographic contraction, most were rare (all but one have frequencies <0.09 in the current populations, range 0.02–0.09). Allele numbers in the relatively small sample size of the prebottleneck population compared to the modern Illinois population strengthen the argument that the prebottleneck population was genetically more variable. Furthermore, the three independent large "control" populations from Minnesota, Kansas, and Nebraska support the idea that the prebottleneck population was genetically more variable given the large number of alleles shared among these populations that are now missing from the modern Illinois population.

The number of alleles and the mean allelic diversity of six microsatellite loci present in the current Illinois, Kansas, Minnesota, and Nebraska populations as well as in the early Illinois birds are shown in table 2. Alleles present in the prebottleneck Illinois population include both currently extant alleles that are shared with the other populations and alleles detected in the museum collection. Including the alleles found in the museum specimens, the mean number of alleles per locus in the prebottleneck Illinois population is not significantly different from that of the present populations in Kansas, Minnesota, and Nebraska, but it is significantly higher than in the current Illinois population (ANOVA, $P < .0051$; Student-Newman-Keuls test, $P < .05$; see table 2). A nonsignificant interaction ($P = .0860$) between population and locus indicated that the number of alleles present at each locus did not depend on the population considered. The permutation test for significant differences in the number of alleles among populations showed similar results. The observed F statistics for both permutation distributions indicated significance levels similar to those from the standard ANOVA ($P < .0054$ and $P < .0036$, assuming additivity and nonadditivity, respectively).

Overall, these results strongly support the idea that the Illinois population originally had higher levels of genetic diversity that were consequently lost through its demographic contraction during the last century. While all components of genetic diversity are affected by small population size, bottlenecks are predicted to have a large effect on allelic diversity (Nei et al. 1975; Leberg 1992), particularly for alleles that are at low frequency. It is also important to point out that although the estimated size for the Illinois population declined from 500 in 1972 to less than 50 in 1993, the effective population size during that period was undoubtedly much smaller given the lek mating system of the greater prairie chicken. Therefore, the potential loss of alleles would be further enhanced by an extremely small effective population size.

In 1974, Bonnell and Selander reported that remarkably low levels of genetic diversity found in the northern elephant seal *Mirounga angustirostrus* (originally measured at the protein level but later confirmed using nuclear [Hoelzel and Le Boeuf 1990] and mitochondrial [Hoelzel et al. 1993] DNA markers) might have been the result of a drastic depletion in numbers caused by human overexploitation during the nineteenth century. Absence of conspecific populations for comparison, however, prevents direct testing of this idea. When genetic data from a sister species (*Mirounga leonina* the southern elephant seal) are used to provide a prebottleneck estimate of genetic variability, demographic and genetic simulations support the idea that the lack of mtDNA variation in *M.*

angustirostrus is the result of a demographic bottleneck (Hoelzel et al. 1993; Hedrick 1995). These simulations are, however, unable to explain the extremely low levels of genetic variation at nuclear DNA markers in *M. angustirostrus* (Hedrick 1995). This illustrates the importance of having an accurate control by assessing directly prebottleneck genetic diversity. Similarly, the absence of genetic variation in the cheetah *Acinonyx jubatus* and the associated decline in reproductive parameters has often been cited as an example of inbreeding depression resulting from one, or possibly even two, putative bottleneck events (O'Brien et al. 1983, 1987). Levels of genetic diversity in the cheetah are low compared to levels in other felid species. Data from conspecific "control" populations are missing, however, and it remains possible that cheetahs are naturally depauperate in genetic variation.

Although the use of "control" populations for comparisons (e.g., conspecific populations that might have undergone different demographic histories) may give general insight into the relationship between genetic diversity and population size in wild species, there is still the potential problem of environmental conditions affecting populations differently at disparate geographic locations. This may result in natural selection favoring a given set of genetic variants at a specific site. For example, a case study (Packer et al. 1991) of a demographic bottleneck in the lions (*Panthera leo*) of the Ngorongoro Crater, Tanzania, compared levels of genetic diversity in this population to those of the larger Serengeti population. However, each population may have been differentially affected by the environmental conditions present at each site (in fact, the Ngorongoro population crash has been considered to be the result of an extraordinary outbreak of biting flies *Stomoxys calcitrans*). Although this problem may be circumvented by increasing the number of independent populations for comparison (e.g., Houlden et al. 1996; Bouzat et al. 1998), only sampling genetic variation from the past (i.e., prior to a demographic contraction) would provide direct evidence for understanding the role that population size may play in the preservation of genetic diversity of natural populations.

To our knowledge, this is the first study providing direct evidence of allelic variants lost from a wild population through a historical demographic contraction. Such a study emphasizes the value of museum collections as possible sources of genetic information from past or extinct populations for addressing questions of ecological/conservation importance.

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