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Low genetic variation in the Heath Hen prior to extinction and implications for the conservation of prairie-chicken populations

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Abstract

Low genetic variation is often considered to contribute to the extinction of species when they reach small population sizes. In this study we examined the mitochondrial control region from museum specimens of the Heath Hen (*Tympanuchus cupido cupido*), which went extinct in 1932. Today, the closest living relatives of the Heath Hen, the Greater (*T. c. pinnatus*), Attwater's (*T. c. attwateri*) and Lesser (*T. pallidicinctus*) Prairie-chicken, are declining throughout most of their range in Midwestern North America, and loss of genetic variation is a likely contributor to their decline. Here we show that 30 years prior to their extinction, Heath Hens had low levels of mitochondrial genetic variation when compared with contemporary populations of prairie-chickens. Furthermore, some current populations of Greater Prairie-chickens are isolated and losing genetic variation due to drift. We estimate that these populations will reach the low levels of genetic variation found in Heath Hens within the next 40 years. Genetic variation and fitness can be restored with translocation of individuals from other populations; however, we also show that choosing an appropriate source population for translocation can be difficult without knowledge of historic population bottlenecks and their effect on genetic structure.

Introduction

The last living Heath Hen (*Tympanuchus cupido cupido*) was seen on 11 March 1932 (Johnsgard 2002). This extinct grouse once inhabited grasslands and barrens along the mid-Atlantic coast of the United States. At the beginning of the nineteenth century, Heath Hens numbered in the tens of thousands and were often harvested for human consumption (Gross 1928; Cokinos 2001). By 1870 Heath Hens were extinct on the mainland, and by 1890 only 200 birds survived on Martha's Vineyard, an island off the coast of Massachusetts (Gross 1928). For the next 40 years conservationists attempted to preserve this population, but it generally remained at less than 500 individuals, and reproduction apparently ceased after 1924 (Gross 1928; Johnsgard 2002).

Today, the closest living relatives of the Heath Hen, the Greater (*T. c. pinnatus*) and Attwater's (*T. c. attwateri*) Prairie-chickens, which are conspecific, and the Lesser Prairie-chicken (*T. pallidicinctus*), are declining throughout most of their range (Schroeder and Robb 1993; Giesen 1998; Johnsgard 2002) (Figure 1). Habitat destruction, overexploitation, disease, and poor reproductive success as a consequence of low genetic variation have all been cited as contributors to the decline and extinction of *Tympanuchus* grouse (Gross 1928; Simberloff 1998; Westemeier et al. 1998). Low genetic variation is often considered to contribute to the extinction of species when

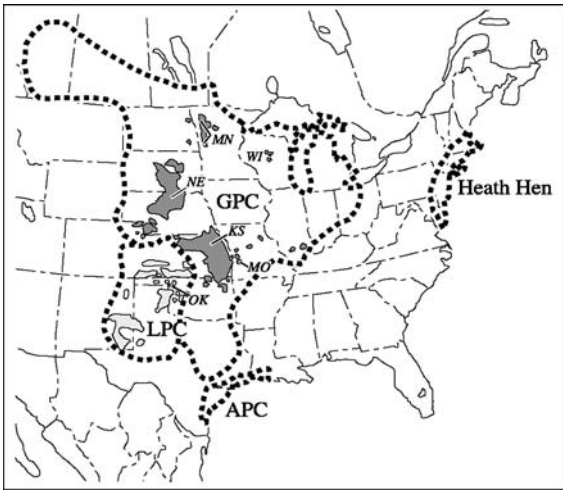

Figure 1. Map of North America showing current and historical (dotted line) distributions for Heath Hens, Greater (GPC), Attwater's (APC), and Lesser Prairie-chickens (LPC). Sample locations are indicated for GPC and LPC, labelled with the state (i.e., Minnesota, MN; Wisconsin, WI; Nebraska, NE; Kansas, KS; Missouri, MO; and Oklahoma, OK).

populations reach small sizes (Newman and Pilson 1997; Reed and Frankham 2003; Spielman et al. 2004). However, the relative roles of genetic, demographic and ecological factors in driving small populations to extinction are controversial (Lande 1988; Mills and Smouse 1994; Lande 1999; Brook et al. 2002; Spielman et al. 2004).

It is acknowledged widely that low genetic variation will often lead to lower growth rates of populations, but it has been suggested that demographic and environmental stochasticity will drive populations to extinction before genetic factors have a large impact (Lande 1988; 1999). To date, relatively few studies of wild populations have examined the loss of genetic variation over the course of a population decline or recovery. In some populations there has been a significant loss of both genetic variation and fitness (Westemeier et al. 1998), but in other cases the results are less clear, as the genetic changes have been relatively small (Miller and Waits 2003), the loss of genetic variation predated the population decline (Mundy et al. 1997; Pertoldi et al. 2001) or the loss of genetic variation was associated with relatively minor effects on fitness (Weber et al. 2000; Groombridge et al. 2000, Bellinger et al. 2003). To date, only two studies of populations in the wild have demonstrated a positive association between genetic variation and the risk of population extinction (Newman and Pilson 1997; Saccheri et al. 1998).

Here we address whether the population of Heath Hens on Martha's Vineyard had low levels of genetic variation using samples collected 30 years prior to their extinction. We examined genetic variation using sequences from the hypervariable region I (HVRI) of the mitochondrial control region and compared it to historic and contemporary populations of Greater, Attwater's, and Lesser Prairie-chickens. These results also provide a benchmark for the conservation of contemporary prairie-chicken populations. In particular, we estimate the loss of genetic variation due to drift, and predict the number of years until contemporary populations of prairie-chickens reach the low levels of genetic variation found in Heath Hens. These estimates are as short as 4 years in some populations of Greater Prairie-chickens in Wisconsin, and they highlight the need for more active management of prairie-chicken populations. Finally, in an attempt to determine which contemporary populations might be suitable for translocation programs, we examine the phylogeography of prairie-chickens. Our analysis reveals a strong effect of genetic drift on estimates of phylogenetic relatedness, and it emphasizes the importance of adequate sampling and the use of historic samples in making conservation decisions.

Materials and methods

Sampling

DNA was extracted from blood and feather tissue of adult prairie-chickens collected from extant populations in one or two adjacent counties in Missouri ($n = 20$), Kansas ($n = 20$), Nebraska ($n = 20$), Minnesota ($n = 40$), and Wisconsin ($n = 80$; see Johnson et al. 2003). For the analysis of historic populations, DNA was extracted from either toe pads of museum specimens (Heath Hens, $n = 21$, and Attwater's prairie-chickens, $n = 19$; see appendix A) or from wings collected during the last Greater Prairie-chicken hunting seasons (1951–1954) in Wisconsin by Fredrick and Frances Hamerstrom ($n = 73$; see Bellinger et al. 2003; Johnson et al. 2004). Historic DNA was extracted following described methods (Fleischer et al. 2000; Lambert et al. 2002) in sets of ten or less using

phenol/chloroform methods followed by a cleaning step using a Qiagen DNeasy kit (QIAGEN) to remove PCR inhibitors.

Mitochondrial DNA sequencing

To reduce the potential for contamination with contemporary prairie-chicken samples, extractions from historic samples were conducted in a new laboratory facility that had not been exposed previously to prairie-chicken DNA, and all Heath Hen samples were extracted and amplified separate from other taxonomic groups. Group extractions (n = 16) were conducted with blank controls and all controls were negative when subjected to PCR. Two primer pairs, 521H (Quinn and Wilson 1993)/186L (5′-CCTTATCCACATTTCTCCCAA-3′) and 272H (5′-TATGTCTATCGAGCATTCAT-3′)/16775L (Quinn 1992) were used to sequence a total of 394 basepairs (bp) from the 5′ region of the mitochondrial DNA control region. PCR was conducted following described methods (Johnson et al. 2003, 2004) with the addition of 1.0 M Betaine to the reaction to improve PCR efficiency. When required, PCR products were amplified a second time to increase concentration necessary for sequencing. Negative controls were included in each PCR reaction. Samples were run on 2% low melt agarose gels and identified bands were extracted using DNAquick Qiagen kits (QIAGEN). Following gel extraction, sequences were obtained using Beckman Coultier TDCS kit and run on a CEQ 8000 capillary sequencer. Multiple extracted samples were replicated providing the same results. Sequence data for the Lesser Prairie-chicken population (n = 62; Oklahoma, Harper & Ellis CO) used in the analyses were provided by Van Den Bussche (see Van Den Bussche et al. 2003), while Blue Grouse (*Dendragopus obscurus*; n = 6) haplotype sequences were obtained through GenBank (AF532426–AF532429, AJ297160–AJ297161).

Statistical analyses

Levels of mtDNA diversity were investigated by comparing population estimates of mitochondrial haplotype diversity (h) and nucleotide diversity (π), and both measures were calculated using the program DNASP v. 3.52 (Rozas and Rozas 1999). Tajima's D estimates were not significant in any population with the exception of the contemporary Paul Olson population in Wisconsin (Table 1; see Johnson et al. 2003), and, thus, most populations conform to neutral expectations.

The loss of genetic variation in prairie-chicken populations was estimated using the standard formula for loss of genetic variation due to genetic drift (Equation (7.15) in Hartl and Clark 1997) modified for the haploid inheritance of mtDNA: $H_t = H_0(1\text{-}1/N_{ef})^t$, where H_t is the expected diversity in generation t due to genetic drift, H_0 is the initial diversity and N_{ef} is the effective population size of females. Estimates of N_{ef} in Wisconsin over the past 50 years were calculated based on two methods. One method estimated N_{ef} as the long-term harmonic mean (1950–1998) of the number of males counted each year on the breeding grounds in each population (Anderson and Toepfer 1999). This method assumed an equal sex ratio and all females mate successfully (Schroeder and Robb 1993).

The second method estimated N_{ef} based on changes we observed in mtDNA haplotype diversity over time, as implemented in the program MLNE (Wang and Whitlock 2003). N_{ef} in Wisconsin was estimated using initial and ending haplotype frequencies from 1951–1954 and 1998–2000, respectively, and a pseudo-likelihood method that incorporates genetic drift and immigration (Wang and Whitlock 2003). The pseudo-likelihood method gives more precise estimates of N_e when migration is present than when it is assumed drift is the only force affecting haplotype frequencies over time (see Wang and Whitlock 2003). We analyzed each of the four Wisconsin populations individually, and for each population we combined the haplotype frequency data from the remaining three populations to serve as a single combined source population that provided immigrants to the focal population (see Johnson et al. 2004). To account for haploid inheritance, values of N_e obtained from the program MLNE were multiplied by two, assuming a diploid population (D) with effective size N_eD will give rise to the same amount of genetic drift (in terms of the variance in gene frequency over one generation), $p(1-p)/(2N_eD)$, as a haploid population (H) with effective size N_eH, $p(1-p)/N_eH$ when $N_eH = 2N_eD$ (Caballero 1994; J. Wang, personal communication). Values of N_{ef} from both methods were then substituted into the formula for genetic drift above to calculate the number of generations (t) until

Table 1. Mitochondrial DNA control region sequence diversity of prairie-chickens

Population	Sample size	Number of haplotypes	Haplotype diversity ($h \pm$ s.e.)	Nucleotide diversity ($\pi \pm$ s.e.)	Tajima's D[a]
Heath Hen (1890–1899)	21	4	0.363 ± 0.029	0.009 ± 0.001	−0.967
Lesser Prairie-chicken[b]	62	22	0.945 ± 0.001	0.014 ± 0.000	−0.428
Attwater's Prairie-chicken	19	12	0.912 ± 0.011	0.009 ± 0.000	−0.162
Greater Prairie-chicken[c]					
Kansas	20	11	0.858 ± 0.015	0.010 ± 0.002	−0.941
Nebraska	20	15	0.968 ± 0.006	0.009 ± 0.001	−1.049
Minnesota-1	20	9	0.847 ± 0.014	0.009 ± 0.001	−1.387
Minnesota-2	20	8	0.889 ± 0.008	0.010 ± 0.001	−0.456
Missouri	20	8	0.842 ± 0.010	0.012 ± 0.001	−0.218
Wisconsin populations					
1951–1954					
Mead	18	11	0.941 ± 0.008	0.010 ± 0.001	−0.669
Paul Olson	19	10	0.860 ± 0.016	0.008 ± 0.000	−0.041
Buena Vista	19	10	0.889 ± 0.013	0.012 ± 0.001	−0.427
Leola	17	9	0.890 ± 0.013	0.012 ± 0.001	−0.046
1998–2000					
Mead	20	3	0.484 ± 0.025	0.010 ± 0.002	1.283
Paul Olson	20	4	0.679 ± 0.017	0.016 ± 0.004	2.744[a]
Buena Vista	20	5	0.511 ± 0.029	0.013 ± 0.003	0.738
Leola	20	6	0.784 ± 0.014	0.014 ± 0.003	1.697

[a]Tajima's D statistic, $P < 0.05$. [b]Original sequence data from Van Den Bussche et al. (2003). [c]Data for Greater Prairie-chicken populations with the exception of Minnesota-2 (Wilken, CO) have been reported elsewhere (Johnson et al. 2003, 2004).

current populations reached the haplotype diversity observed in Heath Hens. We multiplied t by 1.6 years (average age of reproduction; Hamerstrom and Hamerstrom 1973) to convert generations to years after present.

We examined genetic distances between sampled populations using the program ARLEQUIN v. 2.0 (Schneider et al. 2000) and Tamura's (1992) model of sequence divergence, which takes into account multiple substitutions per site, different substitution rates between transitions and transversions, and unequal nucleotide frequencies. Neighbor-joining phenograms were then constructed using PAUP*4.0b10 (Swafford 2002).

The relationship between mtDNA haplotypes was visualized with a minimum spanning cladogram, or parsimony network, estimated using the program TCS v.1.13 (Clement et al. 2000) that provides the 95% parsimoniously plausible branch connections between haplotypes. Phylogenetic analyses among unique mtDNA sequences were also calculated using neighbor-joining (NJ) analyses in PAUP*4.0b10 (Swafford 2002) with the substitution model K81 (Kimura 1981) and a shape parameter (α) of 1.1238 assuming 71.6% invariable sites estimated from the sequence data using the program MODELTEST v.3.06 (Posada and Crandall 1998). The tree was rooted with haplotypes from blue grouse, and a 50% majority-rule consensus tree based on 1000 replicated trees was generated to assess statistical support for nodes defining relationships among haplotypes.

Results

The Heath Hen population from Martha's Vineyard had low levels of mitochondrial genetic variation. Using a standard t-test (Nei 1987), the Heath Hen population possessed significantly lower haplotype diversity ($h \pm s.e. = 0.363 \pm 0.029$) in the mtDNA control region than contemporary populations of Greater ($h = 0.842 \pm 0.010$, $P < 0.01$) and Lesser ($h = 0.945 \pm 0.001$, $P < 0.01$) Prairie-chickens, and historic Attwater's Prairie-chickens collected between 1884 and 1945 ($h = 0.912 \pm 0.012$, $P < 0.01$; Table 1).

Some contemporary populations of prairie-chickens are approaching the low levels of genetic variation found in Heath Hens. Haplotype diversity in Wisconsin populations of Greater Prairie-chickens has declined significantly ($P < 0.001$) over the past 50 years, concurrent with a 73% decline in population size (see Johnson et al. 2004). Haplotype diversity averaged 0.900 (± 0.003; n = 73) in 1951–1954 and 0.641 (± 0.006; n = 80) in contemporary populations (1998–2000). Haplotype diversity was lowest ($h = 0.484$) in the Mead population (Table 1), which is still significantly greater than our estimate of haplotype diversity in Heath Hens (paired $t_{28} = 3.16$, $P < 0.01$).

Based on differences in haplotype diversity between the two time periods and its rate of decline (0.2–0.9%) per year in Wisconsin, the Mead population will have a level of haplotype diversity similar to Heath Hens within 13 years. Estimates of the harmonic mean effective size of females (N_{ef}) based on the pseudo-likelihood method and yearly census counts were 8 (credibility interval = 6–18) and 87 females, respectively. Using estimates of N_{ef} of 8 and 87, the Mead population will reach the haplotype diversity observed in the Heath Hen population in 4–40 years, respectively, and other Wisconsin populations will reach it in 4–117 years (assuming no mutation and a constant N_{ef} of 8 and 216 females).

Four haplotypes were observed among the 21 Heath Hens surveyed. Three of the four haplotypes were unique to Heath Hens and formed a monophyletic cluster that was five to seven fixed nucleotide differences from the closest prairie-chicken haplotype (Figure 2). Interestingly, two Heath Hen samples had a single haplotype that was also found in contemporary Greater and Lesser Prairie-chicken populations (see Figures 2 and 3). This haplotype differed from the three unique Heath Hen haplotypes by at least 10 fixed nucleotide differences, and based on the haplotype network, this single shared haplotype was at least twenty point-substitution steps away from the cluster of unique Heath Hen haplotypes (Figure 2). In a NJ phylogram, the three unique Heath Hen haplotypes formed a cluster (>60% bootstrap support) located at the base of the phylogram that generated a paraphyletic group with a large number of Lesser Prairie-chicken haplotypes (Figure 3). In a Bayesian haplotype tree, the Heath Hen clade (with 0.80 posterior probability) was also observed close to the base of the phylogeny (data not shown), similar to the NJ analysis.

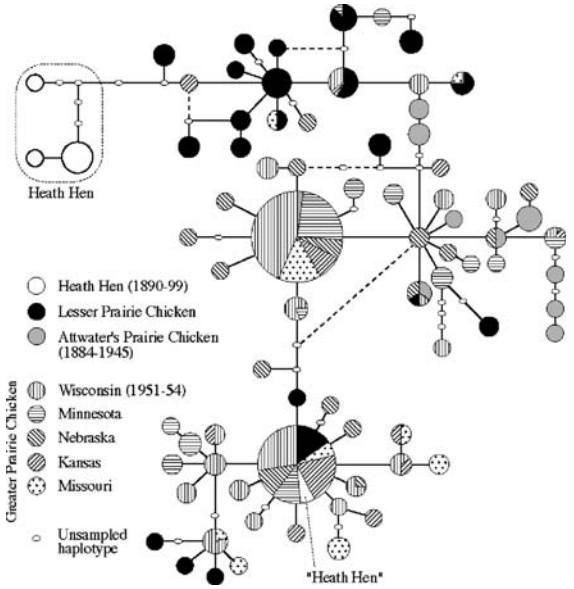

Figure 2. A minimum spanning cladogram inferred from maximum parsimony. Each circle represents a single haplotype where the size of the circle corresponds to the number of individuals observed with that particular haplotype. Solid and dotted lines represent parsimonious connections, between haplotypes with a probability higher than 95%. Dotted lines represent less probable connections based on frequency of similar haplotypes and geographic proximity within network. Each connection between circles corresponds to a single point-mutation, and open circles represent intermediate haplotypes missing in the sample.

Likewise, based on population genetic analyses, the Heath Hen population on Martha's Vineyard appeared more closely related to a Lesser Prairie-chicken population, currently considered a separate species, than to populations of Greater Prairie-chicken with which it is considered conspecific. For example, in a NJ phenogram based on population genetic distances, the Heath Hen population was closer to a Lesser Prairie-chicken population than to the majority of Greater Prairie-chicken populations (Figure 4). However, the placement of the Heath Hen population on the phenogram was probably influenced by genetic drift, as contemporary Greater Prairie-chicken populations in Wisconsin were all closer to a Lesser Prairie-chicken population from Oklahoma than they were to the same Wisconsin population sampled 50 years ago (Figure 4).

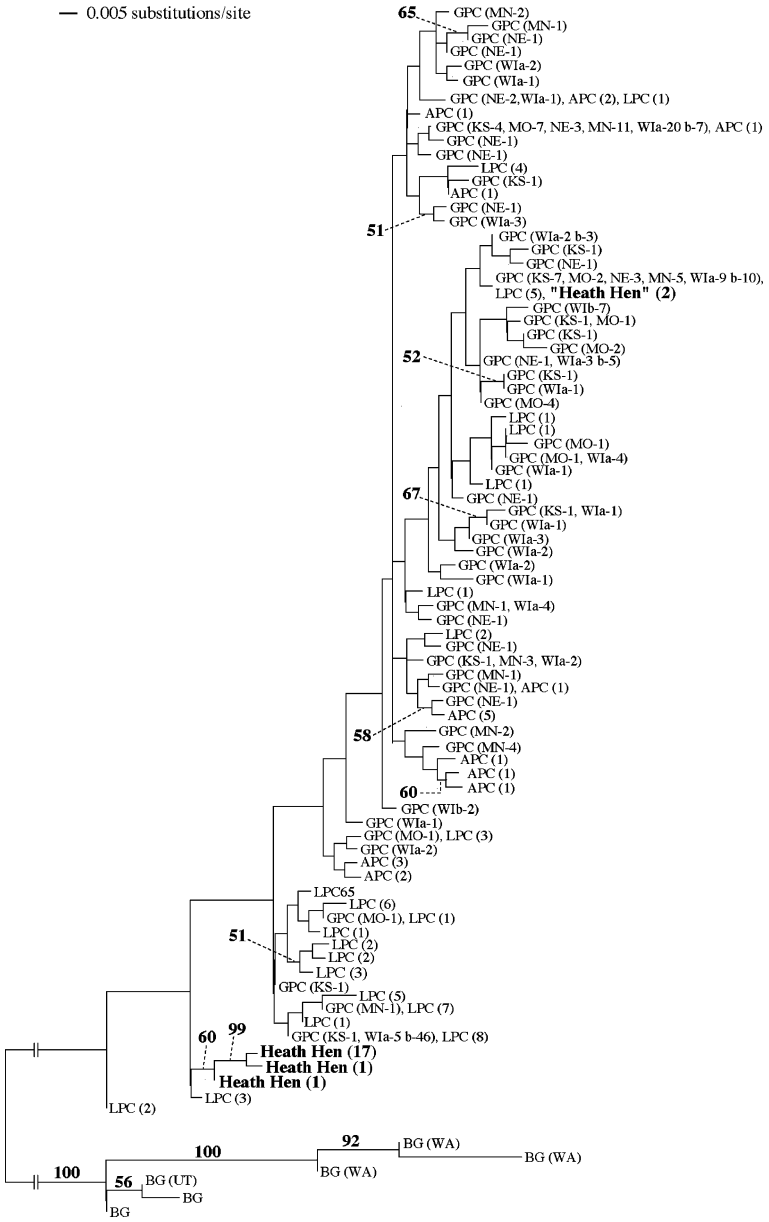

Figure 3. Neighbor-joining phylogram of mtDNA control region sequence variation based on Kimura 3-parameter (K81) substitution model with a shape parameter (α) of 1.1238 assuming 71.6% invariable sites and rooted with Blue Grouse (BG). Abbreviated haplotype names correspond to Greater (GPC), Attwater's (APC), and Lesser Prairie-chickens (LPC). The geographic location and numbers of individuals identified are indicated in parentheses after each haplotype name. Wisconsin GPC individuals are either indicated with an "a" or "b" corresponding to the 1951–1954 or contemporary sampling period, respectively. Branch lengths reflect

Discussion

Thirty years prior to their extinction, Heath Hens had low levels of mitochondrial genetic variation, as would be expected from genetic drift in a small population (population size was <200). These results are consistent with a recent meta-analysis that found small populations of threatened species have lower genetic variation than populations of related species that are not threatened (Spielman et al. 2004). Although it is too late to save the Heath Hen, we can use our results to evaluate the

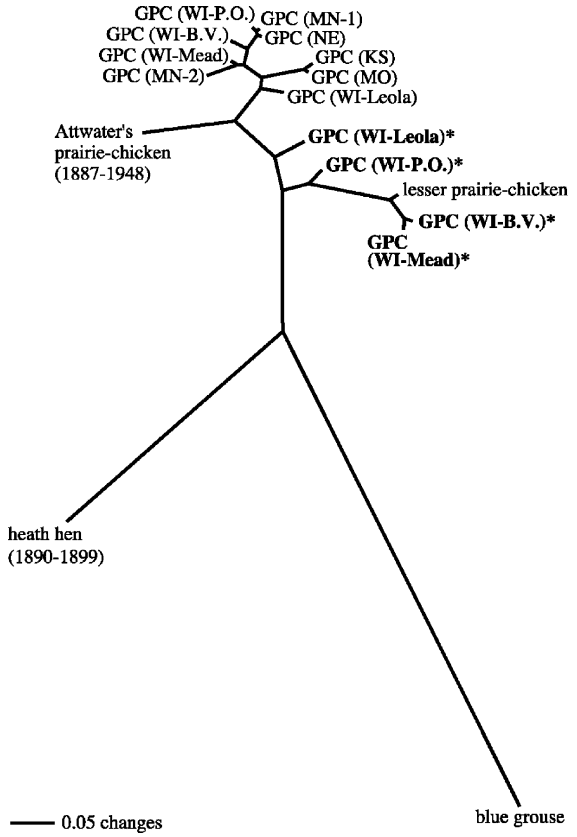

Figure 4. Neighbor-joining phenogram based on Tamura's model of sequence divergence. Branch lengths reflect *population genetic distance* according to scale. Bold labels and "*" indicate contemporary Wisconsin Greater Prairie-chicken populations, which include Mead, Buena Vista (B.V.), Paul Olson (P.O.) and Leola. These same Wisconsin populations in 1951–1954 are in plain font. Labels correspond to those given in Figure 1.

current status of extant prairie-chicken populations. Based on the standard formula for the loss of genetic variation due to drift, we predict that some populations of prairie-chickens in Wisconsin will reach the low levels of genetic variation found in Heath Hens in as little as 4 years. Populations in other states appear to be maintaining higher levels of genetic variation. Interestingly, our phylogeographic analysis of mtDNA sequences revealed a strong effect of genetic drift, which suggests that similar analyses need to consider the demographic history of sampled populations.

Few studies have attempted to predict the future loss of genetic variation in wild populations (Pichler and Baker 2000). We estimate that some contemporary populations of prairie-chickens in Wisconsin will reach the low levels of genetic variation found in Heath Hens within 4–40 years. Haplotype diversity has declined in Wisconsin from a state-wide average of 0.900 in 1951–1954 to 0.641 in contemporary populations (1998–2000). This decline has also been observed at microsatellite DNA loci (Bellinger et al. 2003; Johnson et al. 2004); however, to date, we have found no evidence of reduced hatching success similar to that observed in prairie-chicken populations in Illinois (Westemeier et al. 1998). Based on the analysis of historic (1951–1954) and contemporary (1998–2000) samples, we estimate that haplotype diversity is declining at a rate of 0.2–0.9% per year in Wisconsin. If this decline continues at a constant rate, the Mead population will have a level of haplotype diversity similar to Heath Hens within as little as 13 years. This estimate assumes haplotype diversity began a constant rate of decline in 1951. We also estimated the change in haplotype diversity as a function of genetic drift by incorporating the harmonic mean of N_e for females over the past 50 years using both pseudo-likelihood methods and census counts. These estimates also indicate that genetic variation in Wisconsin populations will reach levels similar to Heath Hens within the next century. These declines may accelerate if there is additional fragmentation of the habitat and isolation of populations within Wisconsin (Johnson et al. 2004).

We were unable to amplify nuclear microsatellite loci with our Heath Hen samples. However, we found previously that levels of genetic variation at both microsatellite and mtDNA markers are lower in populations of prairie-chickens that have been significantly reduced in size (Bellinger et al. 2003; Johnson et al. 2004). Thus, we have empirical evidence to support our use of this marker to examine loss of genetic variation (Johnson et al. 2003, 2004). Indeed, in species such as prairie-chickens with female biased dispersal and a high variance in male mating success (i.e., lek breeding behavior), the effective size of nuclear markers can be significantly smaller than haploid markers (Chesser and Baker 1996; Ballard and Whitlock 2004), and, therefore, our results may be conservative given that a significant reduction in population size can have a larger effect on nuclear than mitochondrial markers (see Johnson et al. 2003).

Similar to other phylogenetic studies of the genus *Tympanuchus* (Ellsworth et al. 1994; Lucchini et al. 2001, Dimcheff et al. 2002; Drovetski 2002;

Palkovacs et al. 2004), we found no resolution of the currently recognized species using maximum parsimony and likelihood techniques (data not shown). These results suggest that speciation within *Tympanuchus* is fairly recent, despite morphological and behavioral differences between species. The molecular divergence among *Tympanuchus* species was less than the intraspecific variation among the six Blue Grouse samples that were used as the outgroup in our analyses (Figure 3).

Interestingly, of the four mtDNA haplotypes observed in Heath Hens in this study, one of the haplotypes was also observed in high frequency among both Greater and Lesser Prairie-chickens. Palkovacs et al. (2004) also documented two Heath Hen haplotypes shared with Greater Prairie-chickens, but they suggested the specimens might not actually be heath hens. In our case, the two male specimens (UMMZ 55259 and 121763) were collected on Martha's Vineyard and they had fewer neck pinnae feathers and more pointed tips on the pinnae than Greater Prairie-chickens (Brewster 1885). Thus, their collection location and morphological features are consistent with Heath Hens. As both studies documented a shared haplotype, it seems less likely that it was due to misidentification. Assuming the shared haplotype is real, it is possible that it was an ancestral and widespread haplotype in *Tympanuchus* grouse that declined in frequency after Heath Hens became restricted to Martha's Vineyard. Alternatively, it is also possible that the shared haplotype represents introgression of prairie-chicken haplotypes into the Heath Hen population on Martha's Vineyard.

After the Heath Hen went extinct on the mainland, multiple attempts were made to establish prairie-chickens in the former range of the Heath Hen (Gross 1928; Phillips 1928). Thousands of Greater Prairie-chickens were translocated from the Midwestern United States to the east coast as early as 1852 (Phillips 1928), yet there are no official records of the release of prairie-chickens on the island of Martha's Vineyard prior to our sampled time period (1890–1899). Records do exist, however, of Heath Hens flying to the mainland (6.5 km) (Gross 1928). Therefore, it is possible that the prairie-chicken haplotype found in our Heath Hen samples entered the Martha's Vineyard population from translocated prairie-chickens.

Heath Hens were probably driven to extinction by a number of factors, including loss of habitat, fires, disease and predation (i.e., foxes, goshawks, rats and feral cats; Gross 1928). Our results indicated that low genetic variation may also have been a contributor, as previously suggested (Simberloff 1988). It is also possible that some of this low genetic variation may reflect the smaller effective population size of island populations (Frankham 1997). Unfortunately, no known samples exist from mainland populations (Gross 1928).

In an analysis of Heath Hen samples from other museums, Palkovacs et al. (2004) reported a significantly higher haplotype diversity ($h = 0.745 \pm 0.024$; $n = 18$) for the mtDNA control region in Heath Hens than we observed ($h = 0.363 \pm 0.029$; $n = 21$). The level of haplotype diversity reported by Palkovacs et al. (2004) in Heath Hens was 8–15% lower than that reported in contemporary populations of prairie-chickens in Minnesota (0.807) and Wisconsin (0.862), while we found that Heath Hens had levels 55–79% lower than in Wisconsin (0.641) and Minnesota (0.840).

Discrepancies between the two studies may be attributed to a number of factors. For example, the Heath Hen samples examined in each study were obtained from different museums, although they were all identified as collected on Martha's Vineyard. Furthermore, some samples ($n = 7$) examined by Palkovacs et al. (2004) had unknown sampling dates, and samples with collection dates ($n = 11$) indicated a wider sampling period (1889–1912) than the samples used in this study (1890–1899).

Third, a detailed comparison of the heath hen sequences obtained in both studies revealed that none of the haplotypes matched, even after controlling for differences in sequence length (± 86 bp); although, the most common haplotype found by Palkovacs et al. (2004; observed in almost half of their samples, $n = 9$), differed from our most common Heath Hen haplotype by only a single nucleotide position. This difference is likely attributable to base-calling variation, rather than additional unique haplotypes. The remaining five unique haplotypes, however, differed from our sequences by 2–6 bp. We have reviewed our chromatograms to assess ambiguities, and feel confident in our results. We have used the same techniques reported in this study to obtain sequence data from >300 prairie-chicken samples from both contemporary and museum specimens without producing conflicting results, and our se-

quences also matched those obtained independently from the same samples (n = 40) by S. Drovetski (personal communication).

The genetic affinities of various populations of prairie-chickens will be an important consideration if prairie-chickens are translocated to restore genetic variation or to provide an ecological equivalent to the Heath Hen on Martha's Vineyard (Palkovacs et al. 2004). Our results suggest that the unique Heath Hen haplotypes were more closely related to a larger number of Lesser Prairie-chicken haplotypes than they were to the Greater Prairie-chicken, its conspecific (Figure 2). In contrast, Palkovacs et al. (2004) found that the unique Heath Hen haplotypes were closer to those of Greater Prairie-chickens from Wisconsin. These differences in the placement of Heath Hens relative to other *Tympanuchus* grouse may be partly attributable to differences in sampling (see Funk and Omland 2003). Our analyses had over three times the number of samples used by Palkovacs et al. (2004), and, more specifically, 15 times (n = 4 versus 60) the number of Lesser Prairie-chicken samples and over seven times the number of Greater Prairie-chicken samples from Wisconsin (n = 21 versus 153). Similar results were obtained when we used analyses based on populations (NJ phenograms; Figure 4) rather than the individual haplotypes (Figures 2 and 3).

The results using population level genetic distances (Figure 4) need to be interpreted cautiously, however, as population bottlenecks and subsequent genetic drift can lead to large increases in genetic distance (Chakraborty and Nei 1977; Hedrick 1999). Indeed, the contemporary populations of Greater Prairie-chicken in Wisconsin were closer to a Lesser Prairie-chicken population from Oklahoma than they were to the same Wisconsin populations sampled 50 years ago (Figure 4). Thus, without information about genetic relationships between populations in the past or knowledge of population bottlenecks, this example shows how researchers could be seriously misled when analyzing phylogeographic relationships and, hence, potential populations for translocations and other types of management.

Unfortunately, there are no Heath Hen samples collected from the mainland (Gross 1928) to determine if their placement closer to Lesser Prairie-chickens (Figure 4) is an artifact of a similar genetic bottleneck or possibly an artifact of a small data set (i.e., few characters) and homoplasy. These results from populations with known demographic histories illustrate that population bottlenecks have a strong effect on genetic affinities (see also Excoffier and Schneider 1999). In these cases, historic samples are invaluable for understanding evolutionary relationships and making appropriate recommendations for conservation.

In summary, we found low mtDNA variation in the Heath Hen population on Martha's Vineyard 30 years prior to its extinction. Thus, low genetic variation may have been a contributor to their ultimate extinction. Unfortunately, over the last 50 years, prairie-chicken populations have declined sharply throughout most of North America, and, loss of genetic variation may be contributing to this decline (Bouzat et al. 1998, Westemeier et al. 1998; Johnson et al. 2004). Our study indicates that within as little as 4 years some current populations of prairie-chickens may reach the low levels of genetic variation found in Heath Hens. Genetic variation and fitness can be restored with translocation of individuals from other populations (Westemeier et al. 1998; Madsen et al. 1999; Keller et al. 2001; Vilá et al. 2003); however, adequate knowledge of phylogeography and demography (population bottlenecks) from historic data sets may be important for choosing an appropriate source population for translocation. As we have shown with historic data from Greater Prairie-chickens in Wisconsin, analyses based solely on contemporary populations may be severely affected by genetic drift and give misleading evolutionary relationships.

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Appendix A. Health Hen and Attwater's Prairie-chicken museum sample information

DNA ID	Taxon	Date collected	Tissue type	County/location	Museum[1]	Museum ID#	Nested primer pairs		Accession number[2]
							521H/186L	272H/16775L	
HH1	T. c. cupido	1894	toe pad	Martha's Vineyard	USNM	203607	+	+	DQ027815
HH2	T. c. cupido	1896	toe pad	Martha's Vineyard	USNM	203608	+	–	–
HH3	T. c. cupido	1896	toe pad	Martha's Vineyard	USNM	203609	+	+	DQ027815
HH4	T. c. cupido	1896	toe pad	Martha's Vineyard	USNM	203610	+	+	DQ027815
HH5	T. c. cupido	1895	toe pad	Martha's Vineyard	USNM	329856	–	–	–
HH6	T. c. cupido	1890	toe pad	Martha's Vineyard	USNM	329857	–	–	–
HH7	T. c. cupido	1898	toe pad	Martha's Vineyard	USNM	462956	+	+	DQ027815
HH8	T. c. cupido	1897	toe pad	Martha's Vineyard	USNM	463322	+	+	DQ027815
HH9	T. c. cupido	1894	toe pad	Martha's Vineyard	UMMZ	55259	+	+	AY273832
HH10	T. c. cupido	1890	toe pad	Martha's Vineyard	UMMZ	55260	+	+	DQ027815
HH11	T. c. cupido	1896	toe pad	Martha's Vineyard	UMMZ	121763	+	+	AY273832
HH12	T. c. cupido	1899	toe pad	Martha's Vineyard	UMMZ	122596	+	+	DQ027815
HH13	T. c. cupido	1893	toe pad	Martha's Vineyard	AMNH	751196	+	+	DQ027815
HH14	T. c. cupido	1895	toe pad	Martha's Vineyard	AMNH	751198	–	–	–
HH15	T. c. cupido	1890	toe pad	Martha's Vineyard	AMNH	751199	+	–	–
HH16	T. c. cupido	1899	toe pad	Martha's Vineyard	AMNH	751200	+	+	DQ027815
HH17	T. c. cupido	1896	toe pad	Martha's Vineyard	AMNH	471694	+	+	DQ027815
HH18	T. c. cupido	1898	toe pad	Martha's Vineyard	AMNH	751197	+	+	DQ027815
HH19	T. c. cupido	1896	toe pad	Martha's Vineyard	AMNH	751203	+	+	DQ027815
HH20	T. c. cupido	1892	toe pad	Martha's Vineyard	AMNH	353622	+	+	DQ027815
HH21	T. c. cupido	1891	toe pad	Martha's Vineyard	AMNH	751202	+	+	DQ027815
HH22	T. c. cupido	1923	toe pad	Martha's Vineyard	MVZ	43878	+	+	DQ027815
HH23	T. c. cupido	1896	toe pad	Martha's Vineyard	MVZ	81904	+	+	DQ027815
HH24	T. c. cupido	1896	toe pad	Martha's Vineyard	MVZ	100273	+	+	DQ027815
HH25	T. c. cupido	1895	toe pad	Martha's Vineyard	MVZ	106775	+	+	DQ027816
HH26	T. c. cupido	1899	toe pad	Martha's Vineyard	MVZ	106776	+	+	DQ027815
HH27	T. c. cupido	1896	toe pad	Martha's Vineyard	MVZ	106777	+	–	–
HTX1	T. c. attwateri	1878	toe pad	Gainesville	USNM	79085	–	–	–
HTX2	T. c. attwateri	1878	toe pad	Gainesville	USNM	79086	–	–	–
HTX5	T. c. attwateri	1893	toe pad	Aransas, CO	USNM	128483	+	+	DQ027820
HTX6	T. c. attwateri	1893	toe pad	Aransas, CO	USNM	128484	–	–	–
HTX7	T. c. attwateri	1893	toe pad	Aransas, CO	USNM	131178	–	–	–
HTX8	T. c. attwateri	1894	toe pad	Jefferson, CO	USNM	132509	+	+	DQ027821
HTX9	T. c. attwateri	1905	toe pad	East Bernard	USNM	197838	+	+	–
HTX10	T. c. attwateri	1905	toe pad	East Bernard	USNM	197839	+	+	DQ027822
HTX11	T. c. attwateri	1905	toe pad	East Bernard	USNM	197840	–	–	–
HTX13	T. c. attwateri	1945	toe pad	Colorado, CO	UMMZ	122597	+	+	DQ027818
HTX14	T. c. attwateri	1948	toe pad	Colorado, CO	UMMZ	122598	+	+	DQ027819
HTX15	T. c. attwateri	1948	toe pad	Colorado, CO	UMMZ	122599	+	+	DQ027824
HTX16	T. c. attwateri	1945	toe pad	Colorado, CO	UMMZ	122600	+	+	DQ027818
HTX17	T. c. attwateri	1893	toe pad	Aransas, CO	AMNH	59538	+	+	DQ027818
HTX18	T. c. attwateri	1911	toe pad	Victoria, CO	AMNH	751219	+	+	AY273865
HTX19	T. c. attwateri	1910	toe pad	Victoria, CO	AMNH	751220	+	+	AY273835
HTX20	T. c. attwateri	1887	toe pad	Matagordon, CO	AMNH	80402	+	+	DQ027823
HTX21	T. c. attwateri	1894	toe pad	Placedo	AMNH	75121	–	–	–
HTX22	T. c. attwateri	1937	toe pad	Colorado, CO	MVZ	100282	+	–	–
HTX23	T. c. attwateri	1938	toe pad	Colorado, CO	MVZ	100283	+	+	DQ027818
HTX24	T. c. attwateri	1938	toe pad	Refugio, CO	MVZ	100284	+	+	AY273865
HTX25	T. c. attwateri	1940	toe pad	Colorado, CO	TX, A&M	1007	+	+	DQ027820
HTX26	T. c. attwateri	1941	toe pad	Colorado, CO	TX, A&M	1008	–	–	–
HTX27	T. c. attwateri	1941	toe pad	Colorado, CO	TX, A&M	1009	–	–	–

Appendix A. Continued

DNA ID	Taxon	Date collected	Tissue type	County/location	Museum[1]	Museum ID#	Nested primer pairs		Accession number[2]
							521H/186L	272H/16775L	
HTX28	T. c. attwateri	1941	toe pad	Colorado, CO	TX, A&M	1010	–	–	–
HTX29	T. c. attwateri	1942	toe pad	Colorado, CO	TX, A&M	1014	–	–	–
HTX30	T. c. attwateri	1941	toe pad	Refugio, CO	TX, A&M	1015	+	+	DQ027818
HTX31	T. c. attwateri	1942	toe pad	Refugio, CO	TX, A&M	1017	–	–	–
HTX32	T. c. attwateri	1942	toe pad	Refugio, CO	TX, A&M	1018	–	–	–
HTX33	T. c. attwateri	1942	toe pad	Austin, CO	TX, A&M	1019	–	–	–
HTX34	T. c. attwateri	1942	toe pad	Colorado, CO	TX A&M	1784	–	–	–
HTX35	T. c. attwateri	1942	toe pad	Colorado, CO	TX, A&M	1785	+	+	AY273857
HTX41	T. c. attwateri	1942	toe pad	Austin, CO	TX, A&M	7654	+	+	DQ027821
HTX42	T. c. attwateri	1941	toe pad	Chambers, CO	TX, A&M	8295	+	+	DQ027820
HTX43	T. c. attwateri	1940	toe pad	Warton, CO	TX, A&M	9670	–	–	–

[1] USNM (Smithsonian, Washington D.C), UMMZ (Univ. of Michigan Museum of Zoology), AMNH (American Museum of Natural History, NY), MVZ (Museum of Vertebrate Zoology, UCA-Berkeley), TX A&M(Texas A&M University).
[2] Only samples where both primer sets amplified product were used in the analyses. Therefore, partial sequences do not have accession numbers.

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