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Rapid morphological and genetic change in Chicago-area *Peromyscus*

OLIVER R. W. PERGAMS* and ROBERT C. LACY†
*Department of Biological Sciences, University of Illinois at Chicago, 845 W. Taylor St., Chicago, IL 60607 USA, †Department of Conservation Science, Chicago Zoological Society, Brookfield, IL 60513, USA

Abstract

We report rapid change of morphology and mitochondrial genes in white-footed mice (*Peromyscus leucopus*) in the Chicago (Illinois, USA) region. We sequenced mitochondrial DNA COX2 from 55 museum skins of white-footed mice caught in the Chicago area since 1855 and from 44 mice recently trapped in the same locations. We found consistent directional genotype replacement at five separate collection locations. We later focused on a single one of these locations (Volo Bog State Natural Area) and sequenced mitochondrial D-loop control region from 58 museum skins of mice collected in 1903–1976 and 32 mice recently trapped there. We found complete and more recent replacement of D-loop haplotypes, apparently occurring between 1976 and 2001. We tested whether these genetic changes were mirrored by changes in morphology by comparing 15 external and cranial traits. We found no significant morphological differences between mice collected in 1903–1976; however, mice collected in 2001–2003 showed 9 of 15 measurements to be significantly changed relative to the earlier samples. Recent mice were longer in total length, with broader, longer noses, and longer but shallower skulls. Discriminant function analysis allowed for 100% correct classification using these traits. Principal components analysis shows variance over time is well distributed across both external and cranial measures. The sequential replacements of haplotypes and the rapid change of morphology can best be explained by replacement of the regional population with immigrants from genetically distinct neighbouring populations, likely facilitated by the large environmental changes occurring over the time period. Replacement with genotypes from external populations may be a common mechanism of evolution of newly adaptive local forms in an increasingly human-impacted world.

Keywords: ancient DNA, Chicago, cytochrome oxidase *c* subunit II (COX2), D-loop control region, microevolution, mitochondrial DNA, morphometrics, *Peromyscus leucopus*, rapid evolution

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Introduction

The study of microevolution is fundamental to understanding the potential rate of adaptation to changed environments, even if macroevolutionary change will usually be much slower or even show stasis because of the averaging of variable rates and direction of microevolutionary change. Darwin (1859) speculated that evolution will usually be so slow that we cannot observe its action except by looking at change that accumulates over the span of many generations.

Correspondence: Oliver R. W. Pergams, Fax: 312 413 2435; E-mail: pergams@uic.edu

Since Darwin, many studies of microevolution have focused on responses to novel toxins and other acute environmental stresses, and have documented rapid development of resistance in bacteria (Lenski & Travisano 1994), insects (Singer *et al*. 1993), and plants (Bone & Farres 2001). Otherwise, however, there have not been many well-documented cases of evolutionary change in natural populations, leading some authors to seek explanations as to why observable rates of microevolution are so rare (e.g. Merila *et al*. 2001). Among vertebrates, most studies of microevolution have focused on change in morphological traits (in fish, reptiles, birds, and mammals) and changes in life-history traits in fishes (Hendry & Kinnison 1999). Other cases of evolutionary

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change in vertebrate morphology over short time periods include alteration of bill shape in island birds in response to climatic changes (e.g. Galápagos finches; Grant & Grant 1993, 2002) or to modification of key aspects of the habitat (e.g. Hawaiian i'iwi; Smith *et al*. 1995).

Studies of island rodents were among the first recognized examples of microevolution (e.g. Clarke 1904; Huxley 1942). It is commonly observed that insular rodents differ from their mainland relatives in a variety of traits, including behaviour, demography, genetics, physiology, and morphology (Gliwicz 1980; Berry 1986; Adler & Levins 1994; Abdelkrim *et al.* 2005). Recent work has further confirmed the generality of increased rates of morphological evolution in island mammals (Millien 2006). Pergams & Ashley (2001) performed a meta-analysis of rapid morphological evolution in island rodents: in *Mus musculus* after introduction to islands of the North Atlantic; in *Rattus rattus* after introduction to the Galapagos Islands; and in *Peromyscus maniculatus* on the California Channel Islands (Pergams & Ashley 1999; Pergams & Ashley 2000). The authors found that microevolution of body size and skeletal traits are greater on smaller and more remote islands. No examples of mammalian morphological microevolution have, to the best of our knowledge, been documented outside of islands.

Largely independent from studies of rapid morphological evolution, recent advances in DNA analyses have allowed examination of rapid evolution at the molecular level. In particular, 'ancient' DNA (DNA extracted from museum specimens and other historical samples) has been utilized over the last 20 years as a tool for phylogenetics using mitochondrial DNA (for a review see Pääbo *et al*. 2004), and more recently as a tool for population genetics using both mitochondrial and nuclear microsatellite DNA (e.g. Roy *et al*. 1996; Nielsen *et al*. 1997; Pertoldi *et al*. 2001; Lambert *et al*. 2002; Hadly *et al*. 2004). Some studies reported changes in haplotype frequencies across time (e.g. Thomas *et al*. 1990; Weber *et al*. 2000), but until Pergams *et al*. (2003), no changes in dominant haplotypes were observed.

Aiding our study was an unusual concentration of museum specimens in the Chicago region. Part of this was due to the fact that there were two major museum collections nearby, those of the Field Museum of Natural History (FM) and those of the Chicago Academy of Sciences (CAS). Museum curators (e.g. R. Kennicott at CAS and C. C. Sanborn at FM) would add to their collections locally, especially with specimens from Volo Bog. Local universities also made periodic, but extensive, contributions.

We here expand on previous study of genetic replacement at the cytochrome oxidase *c* subunit II (COX2) mitochondrial gene that was published as a *Nature* Brief Communication (Pergams *et al*. 2003). We also present new work focusing on the single location with the largest museum sample, Volo Bog. Using an expanded subset of mice, we examine another mitochondrial gene, the D-loop control region, and report accelerated genetic replacement. We also compare morphology of Volo Bog mice. We further report here rapid evolution in cranial and external morphology, largely synchronous with genetic change in mitochondrial DNA haplotypes.

Methods

Samples

Locations for the initial study of mitochondrial haplotypes were identified by examining 18 museums for *Peromyscus leucopus* collected in the six-county Chicago area and finding locations with multiple specimens in collections. Details of this process and the site characteristics are given in Pergams & Nyberg (2001). For the COX2 study (Pergams *et al*. 2003), collection years of the 55 museum specimens spanned from 1855 to 1994. Historical collecting areas that still had natural communities were Illinois Beach State Park, Volo Bog, Highland Park, Glenview, and Palos Forest Preserve, and the COX2 study included an additional 44 mice trapped from these localities in 1999. Locations and sample sizes for all specimens used in our analyses of mitochondrial haplotypes and morphology are listed in Table 1.

For the study of an additional mtDNA locus (the D-loop control region) and morphology, we made use of all museum specimens we could find from the Volo Bog location, supplemented with recent collections by us. Through examination of museum tags and collector notes, all museum specimens from Volo Bog were determined to have been collected within a 2-km^2 area within Volo Bog State Natural Area (Lake County, Illinois, USA). Trapping during 2001–2003 was conducted within this same area. A total of 115 adult *Peromyscus leucopus noveboracensis* mice from the site in Volo Bog were examined: 59 specimens with skin and/or skull preparations held at the Field Museum of Natural History (Chicago, Illinois, USA) and 56 mice caught in 2000–2003 by us. This includes 24 mice caught in spring and summer of 2000 from a broader area (including most of the Volo and adjacent Pistakee Bog State Natural Areas) to evaluate restricted sampling area and seasonality as possible causes of reduced morphological variation observed in the mice caught in Volo Bog in fall of 2001–2003. For the Volo Bog D-loop and morphology studies, collection years spanned from 1903 to 2003 (Table 1). Not all of the Volo Bog mice sequenced earlier for COX2 were also sequenced for D-loop, because we were unable to obtain some of the additional museum samples needed. Similarly, while most of the Volo Bog mice sequenced for COX2 were analysed for morphology, skull damage or lack of external measures prevented us from being able to obtain morphological measurements on all the museum specimens that were sequenced for COX2.

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Table 1 Samples used in this study, categorized by time class and collecting locality. Sample size in each time class is given for COX2 (C), D-loop (D), and morphology (M)

Location	Location coordinates	Time class*				
		1855	1903–1906	1928–1939	1973–1976	1999–2003†
Volo Bog	42°21′N, 88° 11′ W		18C	8C	17C	28C
			15D	30D	13D	32D
			16M	28M	10M	35M
Highland Park (Heller Nature Preserve)	42°13′N, 87°52′W		1C	1C		5C
Highland Park (Highmoor Nature Preserve)	42°12′N, 87°51′W					
Glenview (The Grove)	42°04′N, 87°52′W	5C				6C
Glenview (Woodworth Prairie)	42°03′N, 87°50′W				1C	
Palos Forest Preserves	41°42′N, 87°51′W			1C	2C	5C
Illinois Beach State Park	42°28′N, 87°48′W			1C		5C

*Five mice were collected as isolated specimens a few years before the indicated time class, but were clustered into the time periods labelled with the years encompassing most of the collection. †1999–2003 rather than 2001–2003 because mice collected in 1999 and sequenced only for COX2 are included in this time class.

Table 2 PCR primers used in this study

Gene	Nest no.	Prime Sequences (5′–3′)	Product in bp
COX2	1	F: CCACATCACCTATTATGGAAGAAC	559
		R: TCAGAACATTGGCCGTAAAA	
	2	F: AATTGTCTTCCTAATCAGCTCCT	496
		R: GCCGTAAAATAGGCCTGGTC	
	3	F: AGCACTATAGACGCCCAGGA	400
		R: TGGATGAAATTGTAGCCTGGTT	
D-loop	1a	F: CCACTCGTGCTCCTCTTCTC	451
		R: TCCTTGACAAACCCCAAAAG	
	2a	F: GCCCATTAAACTTGGGGGTA	378
		R: CCTANTTATCCATCAAACCCCC	
	3a	F: CTAACTTGAAACTTTATCAGGCATC	260
		R: GCTTCTGGATCCACTATACAATT	
	1b	F: CCACTCGTGCTCCTCTTC	593
		R: TTAAAATCACTTAACACTGGTGGTA	
	2b	F: GCCCATTAAACTTGGGGGTA	530
		R: CACTCCCCTCATATGCTTTTT	
	2c	F: GACTCACCATCGCCGTAAAG	260
		R: TTCAGGCTAACACGCATTCT	

Genetics

DNA was extracted from fresh samples using DNeasy Tissue Kits (QIAGEN). Museum skins were sampled as follows. A 1.5 mm × 12 mm strip was removed from edge of the ventral seam. Since we found that proteins produced from hair during the Chelex extraction interfered with the polymerase chain reaction (PCR) process, we shaved the strips of skin before proceeding with DNA extraction. Each strip was then minced into 1.5 mm^2. Museum DNA extraction was performed using a Chelex 100 protocol (Walsh *et al*. 1991) modified by the addition of proteinase K (as in Steinberg 1999), with 10 μL of 20 mg/mL proteinase K solution added to 490 μL of 5% Chelex solution. As reported in Pergams *et al*. (2003), we found that repeated freezing and thawing of the supernatant separated out residual proteins and other substances that seemed to inhibit PCR.

We took the supernatant remaining after the freeze/thaw process and subjected it to phenol–chloroform extraction (Maniatis *et al*. 1982). The DNA pellet was re-suspended in 20 μL of 1 mM TE buffer. Amplification was performed by PCR using two sets of three nested primers each. Primers and product sizes are given in Table 2. We performed one or two sets of consecutively nested PCRs, moving in 25–50 bp from the ends of each previous segment, and using the previous PCR product as template. All primers were designed using the program PRIMER3 (Rozen & Skaletsky 1998). Important aspects of our protocols were the use of Klen*Taq* LA DNA polymerase and PCR buffer (DNA Polymerase Technology, Inc.), extremely long (20 min) extension cycles, and use of betaine (Barnes 1994; Cheng *et al*. 1994). Bovine serum albumin was added to further prevent proteins from inhibiting PCR (Pääbo *et al*. 1988). PCR product from all sources was cleaned with QIAquick PCR Purification Kits (QIAGEN) and quantified using a GeneQuant RNA/DNA Calculator spectrophotometer (Amersham Pharmacia Biotech). Sequencing was performed on an ABI PRISM 3100 Genetic Analyser at the University of Illinois at Chicago DNA Sequencing Facility.

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Sequences were aligned with the program CLUSTALW (Higgins *et al*. 1994). The program DNASP 4.10.9 (Rozas *et al*. 2003) was used to calculate: nucleotide diversity π (Nei 1987), the average number of nucleotide differences per site between sequences; haplotype diversity h (Nei 1987), describing the number and frequency of different haplotypes in the sample; and θ per sequence from S (Watterson estimator, Tajima 1993) for mtDNA. This last measure of diversity incorporates the most information of the metrics used. DNASP was also used to calculate genetic differentiation between time classes, using the sequence-based statistic F_{ST} (Hudson *et al*. 1992, eq. 3).

Given our finding of haplotype replacements, we wished to assess if the current distribution of haplotypes was consistent with a constant population size or instead indicated that the genetic replacement came from a population that had expanded from a smaller founder population. Using DNASP, we estimated and graphed observed vs. expected values of pairwise nucleotide site differences (mismatch distribution) in both a population with constant population size (Watterson 1975; Slatkin & Hudson 1991, eq. 1; Rogers & Harpending 1992, eq. 3) and in a nonstable population (Rogers & Harpending 1992, eq. 4; Rogers 1995).

Morphology

Eleven cranial measurements were taken following the methods described in Collins & George (1990) unless otherwise indicated: alimentary toothrow (AL), breadth of braincase (BB), breadth of rostrum (BR), depth of braincase (DBC), greatest length of skull (GL), interorbital breadth (IB), length of braincase (LBC), length of incisive foramen (LIF), length of palate plus incisor (LPI, measured as the greatest distance from the anterior edge of the alveoli of the incisors to the mesopterygoid fossa), length from supraorbitals to nasals (LSN, measured as the least distance from the supraorbital notch to the tip of the nasals), and zygomatic breadth (ZB) (Fig. 1). All cranial measurements were taken by O.P. with a digital caliper, to the nearest 0.05 mm. The four standard external measurements in museum specimens were originally made by seven different museum preparers and recorded from museum tags: total length (TL), tail length (Tail), hind foot length (HF), and ear length (Ear). Because of either lack of external measurement by museum preparers or damage to the skulls, some measurements were not available for many of the 89 specimens (Table 1). All measures available to us were utilized.

Measurement error in cranial measures was estimated by repeating 10 times the 11 cranial variables for each of three randomly chosen mice. We apportioned the variation among measurements and among mice using a nested ANOVA. STATVIEW version 5.0.1 (SAS Institute *et al.* 1998) was used for MANOVAS, and SYSTAT version 11.0 (SPSS, Inc. 2004) was used for all other statistical analyses. Normality was determined by visual inspection of normal probability plots, following the method of Afifi *et al*. (2004). To evaluate sexual dimorphism, two-sample *t*-tests were performed, after dropping six specimens of unknown gender. The specimens of unknown gender were included in subsequent analyses.

To determine if morphological change had occurred, data were categorized into two sets of time classes. One set of four time classes reflected the groupings of collection years of museum specimens: 1903–1906 (n = 16), 1928–1939 (n = 28), and 1974–1976 (n = 10). Another data set was formed to test whether a replacement of D-loop haplotypes found after 1976 was mirrored in morphology by combining these first four time classes (1903–1976, n = 54) and comparing them to 2001–2003 (n = 32). Separately, we also compared the mice caught in 2000 (n = 24) with the 2001–2003 mice in order to evaluate whether reduced morphological diversity in the 2001–2003 mice could have been due to restricted sampling area or seasonality.

We performed two-sample *t*-tests on each individual character, grouped by time class, within each of the two data sets. For example, each of the 15 characters available for specimens in 1903–1906 was compared to the measures of these characters available for specimens in 1974–1976, etc. Sequential Bonferroni adjustments (Rice 1989) to probability levels were performed for all tests. We examined these Bonferroni-adjusted multiple *t*-tests in addition to MANOVA on the full set of characters because we found that the varying and rather frequent damage to the museum skulls did not permit inclusion of the full multivariate data on many of the specimens.

We performed MANOVAS using two sets of traits: (i) using all 15 traits if possible, and (ii) using only those eight traits found significant by Bonferroni-corrected *t*-tests (allowing more specimens to be included than for the analysis of all traits). We also performed separate MANOVAS using different groupings of time classes: one MANOVA compared each of the first four time classes with each other, the other MANOVA lumped the first four time classes (1903–1976) together and compared them with the 2001–2003 time class. Pillai trace is presented as well as the more common Wilks' λ, as Pillai trace is thought to often be more robust to a variety of data parameters (Zar 1999).

To assess changes in both size and shape over time, we performed a principal components analysis (PCA). Although all measurements used the same units (millimetre), the order of magnitude difference between external and cranial measures indicated a more useful PCA if the analysis were carried out on the correlation matrix rather than the covariance matrix, thereby avoiding having the external measures of body size dominate the PCA factors and obscure contributions of the skull morphology. Pairwise deletion was chosen to minimize loss of sample sizes because of missing character data. We chose the number of

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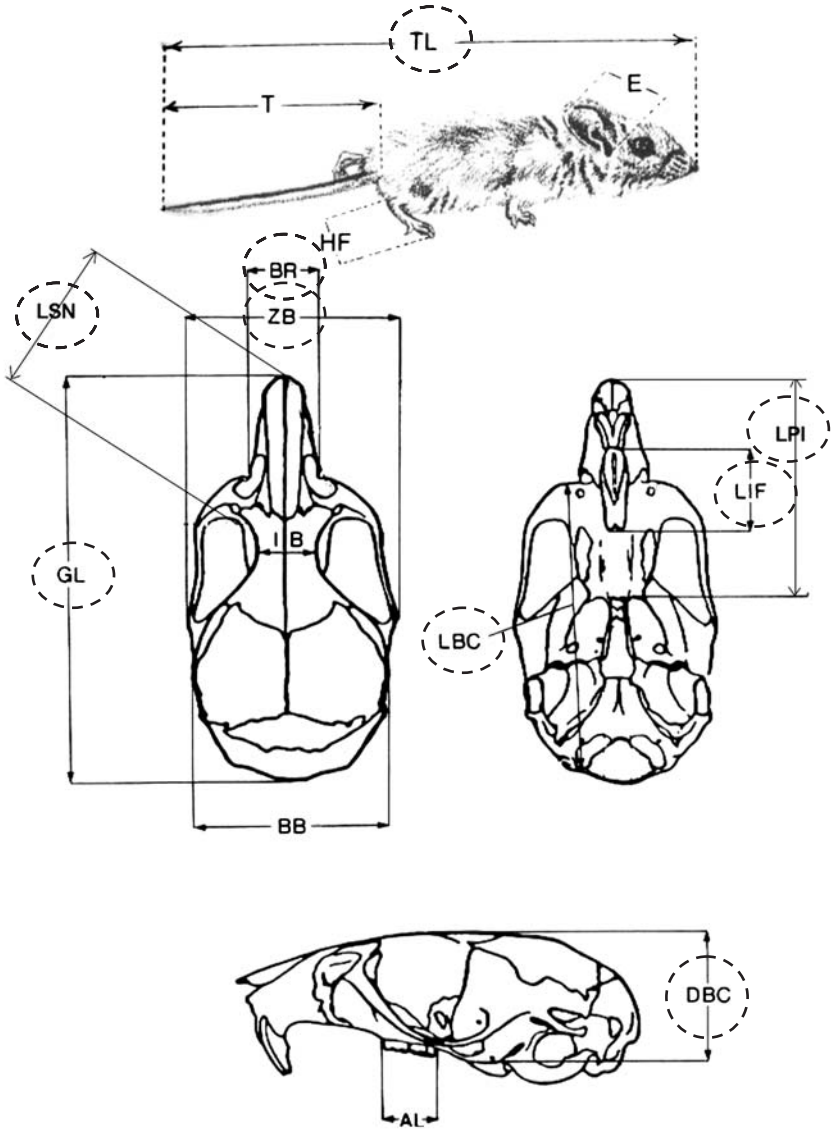

Fig. 1 Morphological measurements taken on *Peromyscus leucopus* mice from Volo Bog. Those that changed significantly are indicated by dashed-line circles.

factors to retain by requiring minimum eigenvalues to equal 1.0, and we used a Varimax orthogonal rotation. To determine which of the factors showed change over time, we compared the individual scores for each of the PCA factors using paired *t*-tests between the two time classes. We also used complete discriminant function analysis (tolerance = 0.001) to determine whether 1903–1976 mice were easily discriminated from 2001 to 2003 mice.

To assess the rate of morphological change, we calculated the rate of evolution in darwins with the equation

$$d = |(\ln x_2 - \ln x_1)/\Delta t|$$

(Haldane 1949), in which x_1 and x_2 are the mean value of each measurement in the time classes 1903–1976 and 2001–2003, respectively, and Δt is time interval per million years. As another measure of evolutionary rate, which is scaled to generation time and variation in the trait, we calculated Haldanes with the equation

$$h = |[(\ln \times 2/s) - (\ln \times 1/s)]/g|$$

in which × 1 and × 2 are means of measurements taken on samples separated by g generations, and s is the pooled standard deviation of ln × 1 and ln × 2 (Haldane 1949). We assumed that the mean generation time for *P. leucopus* in northern Illinois is about 6 months, with breeding females consisting of a mixture of mice born the previous year and mice born early in that breeding season (Millar 1989).

To compare morphological variation between the various time classes, we calculated the coefficient of variation within each time class for each measurement, and then further calculated the mean of coefficients of variation for all measurements within each time class.

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Table 3 Distribution of composite COX2/D-loop haplotypes by time class for those samples sequenced for both genes. The first letter in the haplotype designation is the COX2 haplotype; the second letter is the D-loop haplotype. There were 16 segregating sites and 20 composite haplotypes in the 749 bp sequenced for 54 mice. Solid lines separate the haplotypes to show the replacement first at the COX2 gene and then among sets of related haplotypes at the D-loop region

Haplotype	Variable COX2 sites	Variable D-loop sites	1903–1906	1928–1939	1974–1976	2001–2003
aB	CAGCG	GGCGTAATGGGTTTAAAT	1			
aC	.....	.A.A...C.........A		3		
aE	.....	...A...C.........A	2			
aF	.....	..TA.............A	2			
aI	.....	...A.............A	1			
aJ	.....	..TA...C.........A			1	
mE	TGA.A	...A...C.........A			6	
mF	TGA.A	..TA.............A	1		1	
mI	TGA.A	...A.............A			2	
mO	TGA.A	...A...CA........A		1		
mwI	TGATA	...A.............A		1		
mP	TGA.A	...A..........CT..				1
mQ	TGA.A	...A...C.A....CT..				5
mR	TGA.A	...A...C......CT..				7
mS	TGA.A	...A..GC......CT..				1
mT	TGA.A	...A...CA.....CT..				4
mU	TGA.A	T..A...C......CT..				1
mV	TGA.A	...A...CA...C.CT..				1
mW	TGA.A	..TA..........CT..				11
mX	TGA.A	.A.A...C......CT..				1

Results

Genetics

Pergams *et al*. (2003) sequenced a section of the COX2 gene in 55 museum and 44 fresh specimens from the five localities (Table 1). There were five segregating sites in the 340 bp that were sequenced (Table 3). All five substitutions were synonymous transitions: three within Leucine, one within Isoleucine, and one within Glycine. Four were third-position, the other first-position redundant within Leucine (CUA/UUA). These defined three haplotypes (haplotype diversity $h = 0.432$), designated *a*, *m*, and *mw* (GenBank Accession nos AY266677–AY266679). Haplotypes *m* and *mw* differed from *a* at four sites, with haplotype *mw* differing from *a* and *m* at an additional site. Both haplotypes *a* and *m* were found at all five locations. Haplotype *a* was the only haplotype found in the single pre-1900 collection, it was the majority haplotype in the 1900–1949 collections, and it was completely absent from the 44 specimens from these sites collected in 1999. Pergams *et al*. (2003) did, however, find a single haplotype *a* individual among eight sequenced individuals from three other sites we trapped in southern Cook County in 1999–2001. Haplotype *mw* was found in three specimens, one collected in 1939 from Volo Bog and two in 1999 at Glenview. In only three out of 16 pairwise comparisons of sequential collections from a locality did the frequency of haplotype *a* increase with time ($P < 0.05$ relative to the null hypothesis of equal likelihood of increase or decrease), and all three cases of increase had only a single individual at the later time period.

The replacement of haplotype *a* occurred more or less concurrently at all locations, within the limits of resolution, but there is some suggestion of geographical pattern. The first appearance of haplotype *m* is at the northernmost site, Beach. The latest detection of haplotype *a* was at the southernmost site. The single live specimen of haplotype *a* caught in 2000 was at a site even further south than the Palos preserves.

Focusing on the Volo Bog site, a 409-bp section of the D-loop region of the mitochondrial DNA was sequenced for all specimens, with all museum specimens successfully sequenced. There were 18 segregating sites forming a total of 24 haplotypes (GenBank Accession nos EU030941–EU030964). Nucleotide differences and distribution of these haplotypes by time class is given in Table 4. The first four time classes (1903–1976) all shared some haplotypes. However, the first four time classes shared no haplotypes with the 2001–2003 specimens. Thus, there was complete genetic replacement of D-loop haplotypes between 1976 and 2001.

Genetic differentiation between D-loop time classes is given in Table 5. The values reflect the replacement of D-loop haplotypes between 1976 and 2003. F_{ST} values between the first three time class ranges are relatively undifferentiated

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Table 4 Distribution of D-loop haplotypes by time class. There were 18 segregating sites and 24 haplotypes in the 409 bp sequenced for 90 mice. A solid line separates the set of haplotypes found in early time classes from the set found in the most recent time class. Also given are the measures of genetic and morphological variation for each time class

Haplotype	Variable sites	1903–1906	1928–1939	1974–1976	2001–2003
A	GGCATAATGGGTTTAAAT	1			
B	...G..............	1			
C	.A.....C.........A		3		
D	....G..C.........A		2		
E	.......C.........A	5	10	8	
F	..T.............A	6	1	1	
G	....GG.C.....C...A		1		
H	.....G.C.....C...A		1		
I	.................A	1	7		
J	..T....C.........A	1		1	
K	.......C...A.....A		1		
L	T......C.........A		2		
M	.......C.AA.....GA		1		
N	.......C.A......GA		2		
O	.......CA........A		2		
P	..............CT..				1
Q	.......C.A....CT..				5
R	.......C......CT..				7
S	......GC......CT..				1
T	.......CA.....CT..				4
U	T......C......CT..				1
V	.......CA...C.CT..				1
W	..T...........CT..				11
X	.A.....C......CT..				1
	Haplotype diversity (h)	0.9111	1.0000	0.6026	0.7945
	Nucleotide diversity (π)	0.0041	0.0055	0.0018	0.0022
	θ	0.0038	0.0057	0.0026	0.0025
	Morph. coeff. of var. (mcv)	0.0597	0.0615	0.0384	0.0315

Table 5 Genetic differentiation of D-loop haplotypes between time classes, using the statistic F_{ST} (Hudson *et al*. 1992, eq. 3) and calculated by the program DNASP (Rozas *et al*. 2003)

Population 1	Population 2	F_{ST}
2001–2003	1903–1906	0.638
2001–2003	1974–1976	0.710
2001–2003	1928–1939	0.642
1903–1906	1974–1976	0.106
1903–1906	1928–1939	0.194
1974–1976	1928–1939	0.029

with a range of 0.029–0.194, while F_{ST} values between these time classes and the most recent are highly differentiated with a range of 0.638–0.710.

Graphs of expected vs. observed distributions of 2001–2003 D-loop haplotypes are given in Fig. 2, for constant and nonconstant population size models generated by DNASP (Rozas *et al*. 2003). The fit is much better to the model that allows for nonconstant size population, with the low number of pairwise differences suggesting that the population with the current haplotypes experienced a population bottleneck in its past.

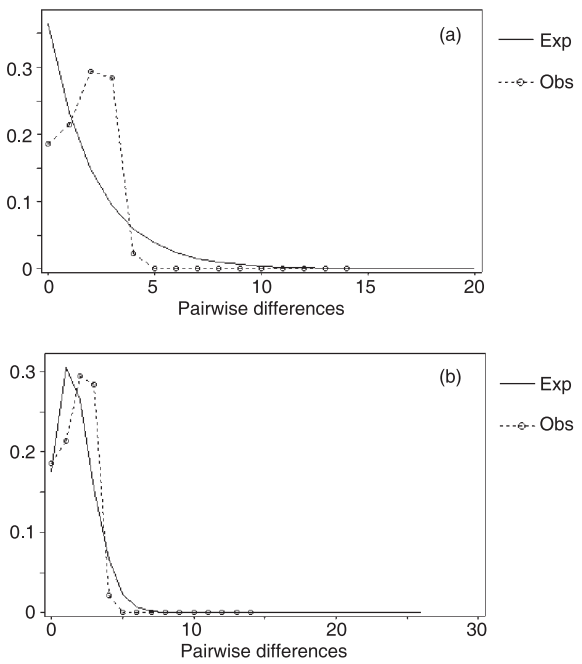

Fig. 2 Observed distribution of D-loop haplotypes in the 2001–2003 collection from Volo Bog compared to those expected from a constant population size model (a) and from a nonconstant population size model (b) generated by DNASP (Rozas *et al*. 2003).

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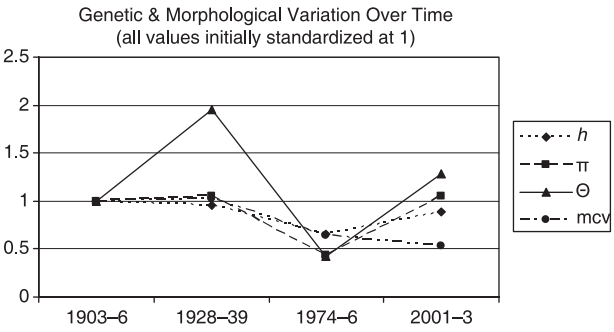

Fig. 3 Changes over time in standardized measures of morphological and genetic diversity for *Peromyscus leucopus* mice at Volo Bog. Genetic diversity is based on D-loop haplotypes.

Nucleotide differences and distribution of composite COX2/D-loop haplotypes by time class are given in Table 3. There were 16 segregating sites and 20 composite haplotypes in the total 749 bp sequenced for 54 mice. The temporal patterns for COX2 and D-loop haplotypes indicate two phases of genetic replacement of haplotypes. The first *m* COX2 haplotype in our data appeared in Volo Bog in 1939, and the last specimen from Volo Bog with the *a* COX2 haplotype was a single individual of the 13 sampled in 1974–1976. The first modern D-loop haplotypes entered Volo Bog sometime between 1976 and 2001, with all mice collected before 1977 having one of 15 closely related haplotypes and all mice collected in 2001–2003 having one of nine haplotypes from a distinct set of closely related D-loop haplotypes.

Figure 3 shows the standardized measures (scaled to 1.0 in the 1903–1906 time class) of each measure of genetic diversity and of morphological diversity. Genetic diversity was lowest in the 1974–1976 time class and returned to prior levels in 2001–2003, while morphological diversity dropped in the 1974–1976 time class and has remained low. The fact that genetic diversity decreased with the 1974–1976 time class suggests that there may have been a declining population that was replaced by individuals (and their descendants) from a different population with a distinct array of haplotypes. The relatively normal (by earlier standards) haplotype diversity among the mice present in the 2001–2003 time class suggests that the replacement occurred with the influx of a number of immigrants, rather than the emergence and diversification *in situ* of a haplotype that was new or previously present but undetected at very low frequency. However, the analysis of the haplotype distribution (Fig. 2) suggests that the immigrant mice had experienced a population bottleneck.

Morphology

Measurement errors for 11 cranial measures (Fig. 1) were estimated to be between approximately 1–4% of the variation among mice, similar in magnitude to those found, for example, in cranial measurements of pocket gophers (Patterson & Patton 1990). Data on the four standard external measurements were single measurements made by original collectors at the time of specimen preparation. Thus, it was not possible for us to estimate error for the external measurements. Measurements of all characters were determined to be approximately normal in distribution, with covariances approximately equal. As a result, and with the exceptions of the calculations of darwins and haldanes, no transformations were made on the raw data. There were no significant differences by gender for any of the 15 measurements. This is consistent with other studies of *Peromyscus* (Rich *et al*. 1996; Sternburg & Feldhamer 1997) in which little or no size dimorphism was found.

There were no significant differences at the 5% level, either before or after Bonferroni adjustment, in any of the 15 morphological characters in 90 two-sample *t*-tests between the first four time classes. However, when we lumped the first four time classes together (1903–1976) and compared them with the 2001–2003 time class, we found that 12 of 15 characters were significantly different before Bonferroni correction, and nine remained statistically different after Bonferroni correction (Table 6). In all but one case of significant change [DBC (depth of the braincase)], the character became bigger over time. In general, the mice grew longer in total length, with broader, longer noses and longer, shallower skulls. However, the 15 morphological traits are not independent, and the observed changes could be due to the measures all increasing with larger overall size or all reflecting a single pattern of change in shape.

MANOVAs corroborate individual results from the *t*-tests. We were not able to compare the first four time classes with each other using all 15 traits, as there was too much skull damage to maintain adequate sample size per time class. However, we were able to compare the first four time classes lumped together (1903–1976) with the 2001–2003 time class using all 15 traits. The mice from 2001 to 2003 were highly significantly different from the earlier populations of mice (Wilks' $\lambda = 0.135$; Pillai trace = 0.865; $F = 10.714$; d.f. = 15, 25; $P < 0.0001$). To allow inclusion of more of the specimens that had some skull damage, we also subjected to MANOVA only those traits found in single trait *t*-tests to be significantly different after Bonferroni correction. A MANOVA comparing the first four time classes showed no significant multivariate differences using these nine traits (Wilks' $\lambda = 0.261$; Pillai trace = 1.062; $F = 1.400$; d.f. = 27, 69; $P = 0.13$). A MANOVA comparing the first four time classes lumped together (1903–1976) to the 2001–2003 time class showed strongly significant multivariate differences using these nine traits (Wilks' $\lambda = 0.465$; Pillai trace = 0.535; $F = 5.763$; d.f. = 9, 45; $P < 0.0001$).

There were no significant differences in two-sample *t*-tests (at the 5% level, either before or after Bonferroni adjustment) in any of the 11 cranial characters between 24

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Table 6 Results of two-sample *t*-tests grouped by time class performed on each cranial and external measurement, with sample sizes, means, standard deviations, and separate variance *t* with associated probabilities (both uncorrected and after sequential Bonferroni correction). Those measurements varying significantly at the 5% level after Bonferroni correction are in bold

Measurement	Time class	*N*	Mean	SD	*t* (uncorr. *P*)	Adjusted *P*
AL	1903–1976	51	3.607	0.127	0.096 (0.924)	1.000
	2001	24	3.607	0.127		
BB	1903–1976	42	10.973	0.406	–2.369 (0.021)	0.126
	2001	23	11.125	0.178		
BR	**1903–1976**	**53**	**4.623**	**0.279**	**–4.731 (0.000)**	**0.000**
	2001	**24**	**4.902**	**0.203**		
DBC	**1903–1976**	**43**	**9.195**	**0.330**	**2.819 (0.007)**	**0.049**
	2001	**23**	**8.966**	**0.254**		
Ear	1903–1976	25	15.950	1.258	–2.185 (0.035)	0.175
	2001	23	16.604	0.778		
GL	**1903–1976**	**41**	**25.375**	**0.940**	**–4.188 (0.000)**	**0.000**
	2001	**23**	**26.086**	**0.432**		
HF	1903–1976	45	20.529	1.016	–2.032 (0.046)	0.184
	2001	23	20.891	0.451		
IB	1903–1976	52	4.013	0.152	–1.154 (0.137)	0.274
	2001	24	4.064	0.152		
LBC	**1903–1976**	**41**	**17.501**	**0.610**	**–3.530 (0.001)**	**0.009**
	2001	**23**	**18.009**	**0.508**		
LIF	**1903–1976**	**52**	**4.166**	**0.533**	**–5.215 (0.000)**	**0.000**
	2001	**24**	**4.750**	**0.381**		
LPI	**1903–1976**	**49**	**13.843**	**0.711**	**–5.363 (0.000)**	**0.000**
	2001	**24**	**14.478**	**0.330**		
LSN	**1903–1976**	**52**	**9.550**	**0.635**	**–3.086 (0.003)**	**0.024**
	2001	**24**	**9.931**	**0.406**		
Tail	1903–1976	45	76.700	7.452	–1.973 (0.053)	0.159
	2001	23	79.674	4.889		
TL	**1903–1976**	**45**	**162.544**	**27.167**	**–4.427 (0.000)**	**0.000**
	2001	**23**	**181.587**	**6.952**		
ZB	**1903–1976**	**40**	**13.030**	**0.584**	**–4.887 (0.000)**	**0.000**
	2001	**24**	**13.589**	**0.279**		

skulls of mice caught from a wider trapping area in spring–summer of 2000 and the 32 skulls of mice caught in Volo Bog in fall of 2001–2003. The mean coefficients of variation of the two groups were similar, with the more broadly sampled 2000 group showing slightly less variation (mcv = 0.021) than the 2001–2003 group (mcv = 0.036, Fig. 3). Thus, restricted sampling area and seasonality were not the causes of a lower morphological diversity seen in the 2001–2003 sample compared to samples from across 1903–1976 time classes.

Principal components analysis allowed explanation of 73.2% of the variance with four factors (Table 7). Factor I (explaining 48.4% of variance) is primarily general size, with all 15 measures loading positively except DBC, which loaded negatively. Ten of these 15 measures have > 0.5 loading, and of these only two are external (TL & Tail). Factor II (9.8%) consists primarily of ear and hind foot length, with contribution from a cranial foramen. Factor III (8.1%) discriminates using primarily braincase depth and breadth. Factor IV (6.9%) has greatest contribution from toothrow length. PCA Factor I describes well the morphological differences between the 1903–1976 and 2001–2003 time classes, with the *t*-test between groups for Factor I scores showing very strong differentiation on this axis (two-tailed $P < 0.001$). The other three PCA Factors did not differ significantly among these time classes.

Discriminant function analysis correctly classified 100% of individual mice into 1903–1976 and 2001–2003 groups. The jackknifed correct classification rate is also very high at 95%. The cumulative proportion of total dispersion = 1.000 and Wilks' $\lambda = 0.135$ (app. $F = 10.715$; d.f. = 15, 25; *p*-tail < 0.0001), indicating an extremely high amount of discriminatory power. Canonical discriminant function coefficients (standardized by within variances) are given in Table 7. While Tail and TL still have the greatest influence, coefficients for GL and IB are of the same order of magnitude, and their coefficients are still > 0.5. Thus, 2001–2003 mice are invariably discriminated from 1903 to 1976 mice, by cranial as well as external measures.

Calculations of rate of evolution are presented in Table 8, for nine traits that showed significant change. The mean time interval used for calculations was 27.5 years, calculated from the midpoint of the 1974–1976 and 2001–2003 time classes. The amount of evolutionary change of traits ranged from 2.5% to 14.0%, and the rate of evolutionary change

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Table 7 Rotated loading matrices for morphological traits from PCA, as well as standardized DFA coefficients for the contrast between 1903 and 1976 vs. 2001–2003 time classes. Values > 0.5 are in bold

	PCA loadings				DFA: 1903–1976 vs. 2001–2003
Measure	Factor I (48.4%)	Factor II (9.8%)	Factor III (8.1%)	Factor IV (6.9%)	Coefficients
Ear	0.010	**0.900**	–0.026	–0.029	0.212
HF	0.287	**0.653**	0.088	0.060	–0.004
Tail	**0.774**	0.224	–0.021	–0.161	**–1.873**
TL	**0.852**	0.273	0.018	–0.170	**2.188**
BR	**0.761**	–0.064	–0.039	0.143	–0.013
ZB	**0.847**	0.334	0.130	0.100	0.493
LSN	**0.785**	0.278	–0.004	0.316	0.028
GL	**0.853**	0.247	0.240	0.211	**–1.282**
BB	**0.518**	0.213	**0.546**	0.217	0.275
IB	**0.609**	–0.147	0.287	–0.232	**0.650**
LBC	**0.812**	0.207	0.209	0.078	–0.278
LIF	0.297	**0.576**	0.254	0.078	0.250
LPI	**0.796**	0.416	0.068	0.302	**1.085**
DBC	–0.017	0.095	**0.913**	0.032	–0.392
AL	0.070	0.030	0.087	**0.895**	-0.239

Table 8 Amount and rates of rapid morphological change, calculated over the 27.5 years interval from the midpoint of the 1974–1976 time class to the 2001–2003 time class

Morphological trait	Percentage of change	darwins (27.5 years)	haldanes (27.5 years)
BR	6.04	2134	0.1060
DBC	–2.50	915	0.0383
GL	2.80	1005	0.0169
LBC	2.90	1041	0.0231
LIF	14.02	4772	0.1249
LPI	4.59	1631	0.0353
LSN	3.99	1422	0.0321
TL	11.72	4029	0.0001
ZB	4.29	1527	0.0413

ranged from 915 to 4772 darwins (geometric mean $d = 1730$) or 0.0001–0.1249 haldanes (geometric mean $h = 0.0213$). This is a conservative estimate of the rapidity with which evolutionary change occurred, because the changes occurred subsequent to the 1974–1976 time class and before 2001.

Discussion

Morphology

We found that during the latter part of the last century, *Peromyscus leucopus* at Volo Bog in the Chicago area are longer in total length, with broader, longer noses and longer, shallower skulls, relative to specimens from that locality sampled throughout 1903–1976. Principal components analysis showed a general trend towards larger size, and also that variance in the mice over time is well distributed across both external and cranial measures. The current population is morphologically distinct from the population previously at that site, as demonstrated by 100% correct classification of early vs. recent time classes through discriminate function analysis. Pergams & Ashley (2001) found similar changes in cranial evolution among mice and rats on the Galápagos and California Channel Islands. The similarity to the present work is striking: 'Examining individual traits, there is a trend towards the nose becoming longer and wider, while the skull becomes shallower, shown by both rats and mice on five different islands' (Pergams & Ashley 2001; p. 245). It is surprising that the rapid cranial changes found previously in two species of rodents on distant islands are essentially identical to those we observed in another species of suburban, non-island rodents. The finding of Pergams & Ashley (2001) that the rate of microevolution was faster on smaller and more isolated islands, and the finding of Millien (2006) that the rate of microevolution was indeed generally faster on islands than mainlands, clearly do not extend to the comparable change we have now found in a large continental population. Rapid morphological changes observed in island populations are sometimes attributed to release from mainland selective pressures (Dayan & Simberloff 1998; Palkovacs 2003), and this could explain the pattern in the

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rodents introduced to the Galápagos. However, this hypothesis cannot explain the parallel changes in California Channel Island deer mice, which have likely been on the islands since the last glacial maximum, and whose morphological changes have been found to occur over the last 100 years. The very similar pattern of change in a non-island, suburban population of white-footed mice would also not be explained by the selective release hypothesis, creating doubt as to whether the pattern first reported in island populations is actually an island phenomenon.

Considering that rodents (including introduced species, island endemics, and a non-isolated population in the middle of an extensive range of an abundant continental species) in three geographically distant and ecologically very different habitats exhibited very similar morphological changes over the past century, perhaps we need to look for global rather than local causes. Are there global phenomena (probably anthropogenic, given the time frame involved) that might have caused these changes? The observed pattern of change does not accord with a postulated trend towards smaller (not bigger) size because of climate warming (Mayr 1963; Smith *et al*. 1995). Before speculating further about possible global changes that could have caused concordant morphological evolution in rodents, the generality of the trend needs to be confirmed from more populations collected from other localities over the last 100 years.

Genetics

We also found rapid evolution at the molecular level. Two sequential replacements of mitochondrial DNA haplotypes occurred, with the second replacement occurring at least approximately concurrently with rapid morphological evolution. At the COX2 gene, across the Chicago region haplotype *m* replaced haplotype *a*, from which it differs by 4 bp. As no intermediate haplotypes were detected in our collection and the number of differences in this 340-bp fragment of the COX2 gene is large, we infer the common ancestor of *m* and *a* lived far in the past and outside our region, rather than the emergence of haplotype *m* having been a recent and local event. As haplotype *m* consistently replaced haplotype *a* at all locations during the time period covered by our samples, we infer haplotype *m* was introduced at least once (but more probably multiple times) into a Chicago area dominated by haplotype *a*, probably between 1850 and 1900. Although it is unlikely that the COX2 *m* haplotype itself conferred a selective advantage over the *a* haplotype that disappeared, the marker may have been carried by a genetically distinct population that moved into the region and displaced the previously resident population due either to some selective advantage in the changing habitat or to genetic swamping if the immigrants were numerically dominant. The possibility that the replacement could have been simply a random process among competitively equal genetic populations seems unlikely given that this replacement occurred concurrently at sites across the region.

The replacement of COX2 haplotypes across the Chicago region over the century before 1976 was followed by a more sudden genetic change in the D-loop control region within a focal population at Volo Bog. Here, there was complete replacement between 1976 and 2001 of a set of closely similar haplotypes with a new set of haplotypes that were similar to each other but separated from the prior ones by several base substitutions. The divergence between the two sets of haplotypes and the diversity of haplotypes present immediately after the replacement again suggests introduction of the new genotypes via immigrants from outside the area rather than local emergence and diversification.

The nucleotide substitutions among the composite haplotypes show a number of cases of homoplasy, because of multiple occurrences or reversions of some substitutions. Therefore, it is not possible to assign with confidence any one of the earlier haplotypes at Volo Bog as the closest ancestor to the latter ones, nor the reverse, and hypothesized phylogenetic relationships among haplotypes cannot be constructed with a series of single-step substitutions. This lack of simple phylogenetic structure in the observed haplotypes and lack of close relationship of the 2001–2003 haplotypes to any of the prior haplotypes at Volo Bog further supports the suggestion that the new set of haplotypes (D-loop sequences P through X) diversified previously in a different geographical population and arrived subsequent to 1976 to Volo Bog within a set of immigrants, rather than having diversified recently and locally from a haplotype (ancestral to this clade) that either recently immigrated to the area or was previously so rare as to be undetected. These hypotheses could be tested by expanding sampling to a much wider part of the very large geographical range of the species to find the geographical and temporal vectors and sources of the *m* COX2 haplotype and the new D-loop haplotypes and morphology.

The period of D-loop haplotype replacement coincides with the period of rapid residential development of the immediately surrounding area. However, it is highly unlikely that any of the mitochondrial haplotypes are causally related to a selective advantage and/or morphological changes. It is much more likely that the new mitochondrial haplotypes are merely markers for population replacement. Such immigrant mice were probably an ecologically, morphologically, and genetically new type that had some significant selective advantage(s) in the changing local environment, and replaced previously resident mice 'marked' with the older haplotypes.

Because we were only able to analyse mitochondrial sequences in the 'ancient' museum specimens, we cannot know if the genetic changes were due to replacement by

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genetically distinct population of males and females or were instead due to introgression via female lineages alone. However, if the complete replacement of mitochondrial haplotypes was not accompanied by comparable changes in the nuclear genome, then the invading mitochondrial haplotypes themselves must have had a selective advantage (although not necessarily because of the mitochondrial genes that we sequenced) in order to have replaced the prior haplotypes in the absence of a demographic replacement that involved both sexes.

Conclusions

We have presented evidence that wild mice in the Chicago area have undergone significant morphological and genetic change over the last 150 years, with the most drastic changes occurring very recently. It was earlier reported that a significant shift in mitochondrial haplotype frequencies occurred across the Chicago region during the century preceding 1976, resulting in a nearly complete replacement at the COX2 gene. Further analysis of historic and recent samples from a single location, Volo Bog State Natural Area, has confirmed the replacement at the COX2 gene and also showed a subsequent replacement of haplotypes (as evidenced by sequences at the D-loop region) and concordant morphological evolution within the past 30 years. These changes could have resulted from evolution within a continually resident local population, with newly predominant genotypes (both at the mitochondrial genome and at whatever genes control the suite of morphological traits that we observed to change) having arisen by recent mutation or having been previously present but very rare, and then favoured by selection.

However, various lines of evidence suggest instead that in some or all of the observed aspects of evolutionary change, the newer genotypes more likely arrived in multiple immigrants that had competitive advantages of some kind over the previously resident genotypes. In the initial replacement at the COX2 gene, a haplotype that was previously absent or rare locally replaced the previously dominant haplotype at multiple places across the geographical region. In the subsequent replacement at the D-loop region at the Volo Bog site, a diverse set of closely related haplotypes rapidly and completely replaced a previous set of closely related haplotypes, with several mutational steps separating the most similar haplotypes between the early and recent sets. The morphological changes observed at Volo Bog occurred over the same time span as the D-loop replacement and mirror the suite of morphological changes that have been observed in rodents on island populations in very different habitats. The reduction in haplotype (and morphological) diversity in the time period just before the recent changes suggests that the population had already declined in size. The distribution of nucleotide substitutions in the current haplotypes is indicative of a population that expanded from a more narrow base. Together, these suggest that the immigrant or otherwise new genetic form either displaced a genetic type that was already in decline, or perhaps expanded into a largely empty habitat subsequent to a decline of the local population.

The observed evolution of genetic and morphological traits at Volo Bog may not have involved adaptive response to selection if the prior population was entirely or nearly eliminated, and an immigrant population that was distinct but perhaps no better adapted to current environmental conditions simply arrived at a fortuitous time to fill the void. However, the complete disappearance of the local population seems unlikely in a protected natural area that had a relatively unchanged habitat but was surrounded by a matrix that was undergoing rapid suburban development. Thus, whether or not there was direct competition between the former and subsequent populations of mice at Volo Bog, a genetic and morphological form of *Peromyscus leucopus* that is adapted to the current environment replaced a form that was already in decline or was outcompeted by the immigrants.

This is consistent with recent past examples of rapid evolutionary and ecological change, which commonly involve invasion from a genetically distinct population (that evolved its adaptations in a different region) rather than response to selection from among genotypes (already represented within the local diversity, as adaptive response is usually portrayed; for reviews see Hendry & Kinnison 1999; Ashley *et al*. 2003). Such invasives may be species, subspecies, or (as in this study) genotypes and phenotypes. Given the current prevalence of human activities that convert and homogenize habitats by replacing native flora, fauna, and even landscape features with alien ones, and also often cause deliberate or incidental translocation of local biotas, replacement with genotypes from external populations may well be a common mechanism of evolution of newly adaptive local forms in an increasingly human-impacted world.

Previous studies of rapid evolution have generally focused on small, often isolated populations subjected to a new or suddenly modified environment (e.g. adaptation to novel toxins, changes after introduction to new habitats, and responses to sudden climatic change). However, the *P. leucopus* in Volo Bog are part of a geographically extensive, contiguous, population of a very abundant small mammal that occupies a variety of habitats and well tolerates habitat disturbances, including at least moderate anthropogenic habitat alteration. Indeed, *P. leucopus* have persisted and even increased in relative abundance while more prairie-restricted species have been largely eliminated from northern Illinois as former prairie and woodland habitats were mostly replaced by dense urban and suburban areas surrounded by intensive agriculture.

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White (1978) estimates that less than 0.1% of original prairie in Illinois is still covered by native vegetation. Pergams & Nyberg (2001, 2003, 2005) document the local loss of prairie mammals *Microtus ochrogaster*, *Peromyscus maniculatus bairdii*, and *Spermophilus franklinii* over this period, both over time in museum collections and at present through a trapping survey. Although *P. leucopus* is associated with woodlands and *P. maniculatus bairdii* is associated with prairies, Pergams & Nyberg (2001, 2005) also document the displacement over time of *P. maniculatus bairdii* by *P. leucopus* even in prairie. They speculate that this may be the result of fire suppression in prairies, but perhaps the evolution of *P. leucopus* is also involved. The period of haplotype displacement and rapid morphological evolution coincides with the period of *P. leucopus* supplanting *P. maniculatus bairdii* from its native prairies (Pergams & Nyberg 2001, 2005). As a woodland species, white-footed mice have arboreal adaptations: their feet are larger, their tails are longer, and they typically nest in trees. They are also bigger, heavier, and are much more of a habitat generalist than are prairie deer mice. The prairie deer mouse nests in underground burrows. It has been thought that fire suppression and the encroachment of woody vegetation into prairies allowed the arboreal *P. leucopus* to establish footholds and eventually exclude the smaller, fossorial *P. maniculatus bairdii*. We further speculate that perhaps this displacement was accelerated by the introduction of new, larger *P. leucopus* genotypes able to out-compete previous *P. leucopus* genotypes as well as *P. maniculatus*.

We close this article with a note of caution. It is standard procedure to sample organisms at a single point in time (the present), and make inferences about phylogenetic and phylogeographical relationships based on such a chronologic snapshot. The existence of such rapid morphological and genetic change puts very significant constraints on the biological inferences that one can make from such genetic patterns collected at a single point in time.

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O. R. W. Pergams is a conservation biologist at the University of Illinois at Chicago and the Field Museum. He views conservation as a problem-focused discipline, and has utilized diverse methodologies to solve various conservation problems. These methodologies derive from biology (molecular genetics inc. ancient DNA, morphometrics, extinction prediction) as well as the social sciences (sociology, economics). He is able to draw on experience from his first career trading various financial instruments, and which he ended as Chairman of the Board of the trading and brokerage firm Chicago Options Associates. During his career in conservation he has received a number of awards, including a Smith Conservation Research Fellowship and a Fulbright Senior Lecturing/Research Award. R. C. Lacy is the Population Geneticist for the Chicago Zoological Society, he serves on the faculty of the Committee on Evolutionary Biology of the University of Chicago, and is Chair of the IUCN/SSC Conservation Breeding Specialist Group. His research interests include experimental studies on the effects of inbreeding and outbreeding; development of computer simulation models for studying the interactions among genetic, demographic, and environmental causes of extinction; conservation action planning for endangered species; and genetic management of captive populations of wildlife.

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