

MITOCHONDRIAL CONTROL REGION VARIATION IN BANK VOLES
(*CLETHRIONOMYS GLAREOLUS*) IS NOT RELATED TO CHERNOBYL
RADIATION EXPOSUREHEATHER N. MEEKS,[†] JEFFREY K. WICKLIFFE,[‡] STEVEN R. HOOFER,[†] RONALD K. CHESSE,[†]
BRENDA E. RODGERS,[†] and ROBERT J. BAKER*[†][†]Department of Biological Sciences, Texas Tech University, Lubbock, Texas 79409-3131, USA[‡]Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, Texas 77555-1110, USA

(Received 10 July 2006; Accepted 19 September 2006)

Abstract—Three previous studies at Chernobyl, Ukraine, documented elevated mitochondrial DNA diversity in bank voles (*Clethrionomys glareolus*) from radioactively contaminated sites. Little evidence was found to link patterns of diversity in contaminated areas to radiation exposure, but the experimental design precluded discriminating among alternative explanations for elevated diversity in exposed groups. Reference sites selected for the studies were relatively distant from contaminated sites and, additionally, were separated from contaminated sites by large river systems; thus, we hypothesized that differences among sites were correlated with geographic isolation rather than with radiation exposure. For the present study, we added three reference sites, which were selected based on minimal radioactive contamination, proximity to contaminated sites, and absence of obvious barriers to dispersal. We hypothesized that neighboring reference sites should exhibit levels and patterns of diversity similar to those of contaminated sites if the previously detected differences were, in fact, caused by geographic isolation. Indeed, levels of diversity in nearby reference sites are comparable to levels in contaminated sites. Additionally, nearby reference sites contain several haplotypes not observed at other study sites. Our results suggest that levels of diversity in contaminated regions are more plausibly explained by ecological and historical factors than by increased mutational pressure resulting from exposure to Chernobyl radiation.

Keywords—Chernobyl Vole Haplotype Diversity Radiation

INTRODUCTION

As a consequence of the Chernobyl Nuclear Power Plant (Chernobyl, Ukraine) disaster in 1986, biotic systems proximal to Reactor 4 experienced radiation doses at concentrations lethal to many resident plants and animals [1,2]. Surrounding regions were blanketed with radionuclides emitted from the reactor core via two major plumes that traveled west and north of the reactor, resulting in a mosaic pattern of deposition in the local environment [3]. Much of the radioactivity in the region has since attenuated through natural decay processes, and the flora and fauna extirpated at the time of the accident have recolonized the exclusion zone. Despite the chronic doses of radiation that resident organisms still receive, the ecosystem is thriving. Studies conducted after 1994 at Chernobyl indicate that species composition in contaminated and nearby uncontaminated areas are similar and that population densities for some species actually are greater within the exclusion zone [4,5]. The apparent vitality of local biota contrasts sharply with the consequences of chronic exposure, as predicted by laboratory toxicity tests in which acute toxicity data commonly are used to model anticipated effects of ultralow-dose-rate chronic irradiation [6–10]. Deterministic models, which do not account for responses caused by dose rate, indicate that even low doses of radiation, such as those experienced by Chernobyl residents, are capable of producing genotoxicity. Studies conducted at Chernobyl have produced equivocal results [11–16]. Research coupling measured internal dose and estimated external dose with genetic effect in native mammals has not

found evidence of increased mutation rates or other deleterious genetic effects predicted by laboratory studies. Discrepancies between modeled and measured effects underscore the high degree of complexity inherent in adequately characterizing the risks of exposure to chronic radiation.

Dose-rate studies indicate that small mammals in the contaminated Red Forest (51°23.040'N, 30°03.780'E) receive chronic radiation doses as high as 86 mGy/d for the duration of their life cycles [1,2], although absorbed doses may vary dramatically among species. If deterministic dose–response models are accurate predictors of biological response to chronic ionizing radiation, exposure levels are sufficient to induce a variety of injurious genetic effects, including increased mutation rates [17], chromosomal aberrations [18], and global genome instability [19]. Because baseline genetic data were not collected for native species before the Chernobyl accident, such effects resulting from radiation exposure must be inferred through comparisons of exposed and unexposed populations.

The bank vole (*Clethrionomys glareolus*) was selected as the model system for the present study both because of its high body burden of radioactivity and because it likely has completed more than 30 generations in the most radioactive regions of Chernobyl [12]. Previous studies have used a variety of end points to assess levels of genetic damage in bank voles environmentally exposed to chronic radiation [20–22]. Of particular interest are three longitudinal genetic studies, conducted from 1995 to 2001 at Chernobyl, examining variation in the hypervariable portion of the mitochondrial DNA (mtDNA) control region [23–25]. Each of these studies documented higher levels of diversity in animals collected from contaminated sites as compared to animals collected from reference

* To whom correspondence may be addressed
(robert.baker@ttu.edu).

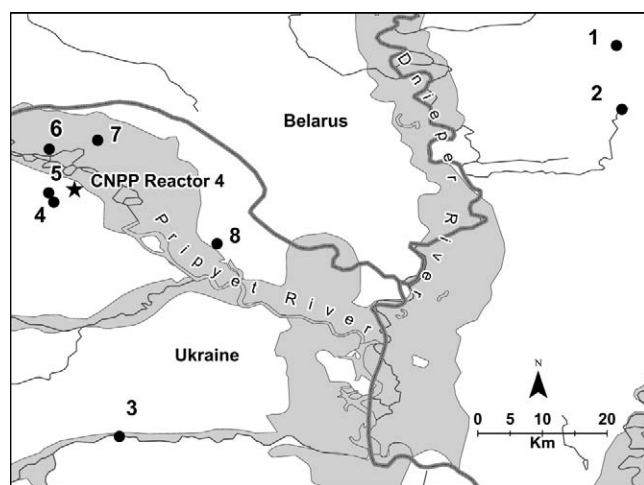


Fig. 1. Map of northern Ukraine. Collection sites for *Clethrionomys glareolus* specimens used in this study are indicated by the numbers 1 to 8. Numbers 5 and 6 are contaminated sites; the remaining numbers indicate uncontaminated sites. 1 = Chista; 2 = Nedanchichy; 3 = Oranoe; 4 = Stupnikovo; 5 = Red Forest; 6 = Glyboke Lake; 7 = Krasnoye; 8 = Paryshev; CNPP = Chernobyl Nuclear Power Plant.

sites. Additionally, more total haplotypes, as well as more unique haplotypes, were identified in mice from contaminated sites than in those from uncontaminated areas.

Two main hypotheses have been proposed to account for these differences in exposed populations. The first suggests that the maternal mutation rate, increased as a function of exposure to low-dose, chronic radiation, is contributing to elevated mtDNA diversity in exposed groups. This hypothesis assumes assortative mating, differences in female-mediated dispersal, and other ecological factors to be negligible contributors to observed differences. The second suggests that levels of diversity in contaminated regions likely result from natural geographic variation in these populations and are mediated primarily by ecological, demographic, and historical processes, such as recolonization in areas where exposure to high, acute doses of radiation led to localized extinction of rodent populations immediately following the Chernobyl accident. Examining distributional differences in haplotype diversity should elucidate which of the proposed mechanisms most likely is associated with the apparently elevated diversity in contaminated sites. If increased diversity is related causally to chronic radiation exposure, then novel haplotypes should be present in exposed populations, and distribution should be restricted to contaminated regions. Additionally, diversity estimates in contaminated regions should be elevated as compared to the diversity in all reference regions, even those in close proximity to radioactive sites. If increased diversity is a product of natural geographic variation, then haplotypes detected in contaminated areas also should be present in nearby uncontaminated areas, and diversity estimates should be comparable. Previous studies [23–25] examining mtDNA diversity were unable to discriminate between the alternative explanations, primarily because study sites were separated by dispersal-prohibitive distances and, in some cases, physical barriers to gene flow. In the present study, we assessed regional variability on a finer scale by sampling uncontaminated localities either in close proximity or adjacent to contaminated sites (Fig. 1), thereby minimizing the confounding effects of variability associated with geographic isolation. We seek to determine whether mtDNA diversity in exposed groups is a

function of ecological processes or is attributable to chronic radiation exposure.

MATERIALS AND METHODS

Experimental design and study specimens

Our experimental design is an extension of previous studies that form a longitudinal genetic monitoring project and includes data from Matson et al. [23], Baker et al. [24], and Wickliffe et al. [25]. The bank vole (*Clethrionomys glareolus*) is the selected model system for the present study, as it was for previous studies. Specimens used for earlier studies ($n = 384$) were collected from the radioactively contaminated areas Red Forest and Glyboke Lake (51°26.695'N, 30°03.826'E) and the reference areas Chista (51°35.335'N, 30°51.138'E), Nedanchichy (51°30.002'N, 30°51.592'E), and Oranoe (51°02.681'N, 30°09.670'E) at Chernobyl, Ukraine, from 1995 to 2001 (Fig. 1). For the present study, we collected specimens during July 2004 from relatively uncontaminated areas in close proximity to radioactive sites at Chernobyl: Krasnoye (51°27.409'N, 30°07.831'E), Paryshev (51°18.805'N, 30°17.799'E), and Stupnikovo (51°22.245'N, 30°04.171'E). The samples sizes for each site were 13, 49, and 22, respectively. We chose these localities specifically to address indeterminate data from previous studies and to elucidate whether unique haplotypes found in voles from contaminated areas were the result of primarily population-level processes or chronic radiation exposure. We harvested liver, kidney, and skeletal muscle tissues from each animal and stored them in lysis buffer [26] immediately after voles were euthanized. The samples collected during the 2004 expedition were intended to augment data sets included in earlier studies conducted from 1995 to 2001. In addition to generating new sequence data in the present study, we have incorporated archived sequences from GenBank® (National Center for Biotechnology Information, Bethesda, MD, USA) to evaluate nucleotide differences among *Clethrionomys* sp. and to verify species identification of our specimens. Sequences used in the present study are archived under GenBank accession numbers AJ236833, AF367179 to AF367201, and AY449642 to AY449649, and they include representatives of the following species: *Clethrionomys glareolus*, *Clethrionomys californicus*, *Clethrionomys gapperi*, *Clethrionomys rutilus*, and *Clethrionomys rufocanus*. Within the context of the present study, we examined a total of 468 *C. glareolus* specimens from northern Ukraine. Sequence data from an additional 23 samples, representing other species of *Clethrionomys*, also were included in the analysis.

Molecular methods

We used standard phenol-chloroform and alcohol precipitation methods to isolate genomic DNA from liver tissue samples. We amplified and sequenced a 291-bp segment of mtDNA (tRNA Proline, 26 bp; control region, 265 bp) according to the methods described by Matson and Baker [27], although we performed sequencing reactions with BigDye® Terminator (Ver 3.1; PE Applied Biosystems, Foster City, CA, USA). We verified and assembled sequences using Sequencing Analysis software (Ver 3.4.1; PE Applied Biosystems) [28] and AssemblyLIGN™ software (Ver 1.09b; Oxford Molecular, Oxford, UK) [29].

Table 1. Polymorphic sites and substitutions in haplotypes from the bank vole (*Clethrionomys glareolus*) and other species of *Clethrionomys* ($n = 492$)^a

Haplotype	Sequence position										
	40	50	56	143	152	153	156	157	170	197	267
1	C	A	T	A	T	C	T	C	C	T	T
2	.	G	T	T	.	.
3	C
4	T	.	.	.
5	.	.	C
6	T	.	.
7	T	.	T	.	.	.
8	C
9	.	.	C	.	.	T	.	T	.	.	.
10	T
11	T	T	.	C	.
12	T	.	C
13	C	T	.	.	.
14	.	.	.	G	C	.	.	T	.	.	.
15	.	.	C	T	T	.	C
AJ236833 Cgl	.	.	C	T	T	C	C
AF367195 Cgl	.	.	C	T	T	.	C
AF367196 Cgl	T	T	.	C
AF367199 Cgl	A	T	.	.
<i>Clethrionomys californicus</i>	T	T	C	.
<i>Clethrionomys gapperi</i>	.	G	C	.	C	A/T	C	A	T	T	C
<i>Clethrionomys rufocanus</i>	A	.	C	T	T	A	C
<i>Clethrionomys rutilus</i>	.	G	C	A/T	T	C	.

^a Haplotype 1 is used as a point of reference. Matson et al. [23] provide detailed information about sequence position. Specimens of *C. glareolus* (Cgl) are listed individually, whereas consensus sequences were generated for other species. C = cytosine; A = adenine; T = thymine; G = guanine.

Data analysis

We assigned sequences to haplotypes using MacClade 4 software (Ver 4.05; Sinauer Associates, Sunderland, MA, USA) [30]. We used Arlequin software (Ver 2.000; Excoffier, Geneva, Switzerland) [31] to compute the Nei diversity index [32], h , as detailed by Matson et al. [23]. We also used Arlequin software to analyze the extent to which variation was partitioned both within and among groups. Sites were assigned to contaminated (Glyboke Lake plus six sampling periods for the Red Forest) or uncontaminated (Chista, Nedanchichy, Oranoe, Krasnoye, Paryshev, and Stupnikovo) groups and were analyzed hierarchically using ϕ -statistics in a nested analysis of molecular variance (AMOVA). We further examined the partitioning of variation in these populations by randomly grouping sites and performing an AMOVA, in a manner similar to that outlined above, for each grouping strategy. We used MEGA software (Ver 2.1; Arizona State University, Tempe, AZ, USA) [33] to generate a minimum evolution tree for evaluating relationships among haplotypes and to verify identification of our specimens as *C. glareolus*, based on Tamura–Nei genetic distances. We generated a map (Fig. 1) of the collection localities using ESRI® ArcMap[®] (Ver 9.1; ESRI, Redlands, CA, USA) [34].

Dose estimates

We estimated internal radioactivity burdens for specimens ($n = 86$) collected from the newly added reference sites Krasnoye, Paryshev, and Stupnikovo. Methods for determining internal radiocesium and strontium levels, as well as methods for estimating dose rates, are detailed by Chesser et al. [1]. Internal radioactivity and dose rates for contaminated sites and other reference sites were estimated in earlier studies [1,2], and these estimates are provided in *Results*.

RESULTS

In previous studies, 11 haplotypes for *C. glareolus* from five localities in northern Ukraine were identified. We identified four novel haplotypes from two of the three reference localities added in the present study. We detected 11 variable nucleotide positions (Table 1). We observed only transition substitutions in haplotypes from the combined data set, and the majority of these (9 of 11) were pyrimidine transitions.

Ukrainian sample analyses

Of the 11 haplotypes detected in the three previous studies, four were unique (i.e., present in only one locality), and three of these four were unique to contaminated sites: Haplotype 2 was found only in Red Forest samples, and haplotypes 9 and 10 were found only in Glyboke Lake samples. Haplotype 11 was unique to the reference site Nedanchichy. Haplotypes 1, 3, 4, 5, 7, and 8 were shared among localities, with at least one contaminated and one reference site represented for each haplotype. Haplotype 6 was detected only in populations inhabiting contaminated regions. With the expansion of the data set to include three new reference localities, the number of identified haplotypes increased to 15. Haplotypes 1, 3, 4, 5, and 6 from the preceding data sets were represented in at least one of the added collection localities, and four novel haplotypes (haplotypes 12–15) also were identified in the additional sites (Table 1). Haplotypes 12, 13, and 14 were found only in Paryshev. Haplotype 15 was found in both Paryshev and Krasnoye. Sequence data for newly identified and unique haplotypes were verified by two individuals, with DNA extractions, amplifications, and sequences generated independently for each sample during the verification process; thus, all haplotypes were considered to be legitimate. Haplotypes 2, 9, and 10, which in previous studies were found only in Red Forest

Table 2. Haplotype frequencies in Ukrainian bank vole samples for all sites, with multiple sampling periods and/or years listed individually^a

Site	Haplotype														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Chista	0.24			0.59	0.06			0.12							
Nedanchichy				0.89				0.07			0.04				
Oranoe 1998	0.44		0.02	0.44			0.09								
Oranoe 1999	0.50			0.36			0.14								
Glyboke Lake	0.50		0.04		0.04	0.29				0.11	0.04				
Red Forest 97	0.42	0.15		0.17	0.24	0.01		0.01							
Red Forest S 98	0.38	0.25		0.25				0.13							
Red Forest F 98	0.43	0.05		0.2	0.08	0.03		0.23							
Red Forest 99	0.43	0.2		0.2		0.05		0.13							
Red Forest 00	0.40	0.27		0.07	0.07	0.07		0.13							
Red Forest 01	0.43	0.39		0.06	0.04	0.02	0.02	0.06							
Krasnoye	0.54		0.15			0.15									0.15
Paryshev	0.23		0.02	0.04		0.59						0.02	0.04	0.02	0.04
Stupnikovo	0.5			0.04	0.23	0.23									
<hr/>															
Chista	0.24			0.59	0.06			0.12							
Nedanchichy				0.89				0.07			0.04				
Oranoe	0.46		0.02	0.42			0.11								
Glyboke Lake	0.50		0.04		0.04	0.29				0.11	0.04				
Red Forest	0.42	0.22		0.16	0.11	0.04	0.02	0.12							
Krasnoye	0.54		0.15			0.15									0.15
Paryshev	0.23		0.02	0.04		0.59						0.02	0.04	0.02	0.02
Stupnikovo	0.5			0.04	0.23	0.23									

^a S = summer sampling period; F = fall sampling period. Radioactive sites are Red Forest and Glyboke Lake, located near Pripyat, Ukraine. Reference sites are Chista, Nedanchichy, Oranoe, Krasnoye, Paryshev, and Stupnikovo. Averages across sampling periods are also noted for each locality, below the dashed line.

or Glyboke Lake, were not detected in the newest samples, and haplotype 11, which was previously identified only in Nedanchichy, also remained unique to that location. All variable nucleotide positions are the result of transition substitutions. Nine of the 11 variable sites have C↔T transitions, and 2 of the 11 sites have A↔G transitions. Five of the 11 variable nucleotide positions contain a polymorphism that is unique to a single haplotype, whereas the remaining six polymorphisms are represented in two or more haplotypes (Table 1).

The frequencies of identified haplotypes for all collection localities are shown in Table 2. Haplotypes 12, 13, and 14, found only in Paryshev, occur at frequencies of 2, 4, and 2%, respectively. Haplotype 15 occurs at a frequency of 4% in Paryshev and 15% in Krasnoye. The most common haplotype for both Krasnoye and Stupnikovo samples was haplotype 1, and the most common haplotype for Paryshev samples was haplotype 6. In previous data sets [25], haplotype 1 was, on average, the most common haplotype identified in Oranoe, Glyboke Lake, and Red Forest. Haplotype 4 was most common in Chista and Nedanchichy.

The Pearson's correlation coefficient (r), which was calculated to determine the degree of association between sample size and number of haplotypes in each sites, was 0.42, indicating a moderate degree of correlation; r^2 reveals that approximately 18% of the variation in the number of haplotypes per site can be accounted for by sample size. Calculating the Pearson's correlation coefficient to estimate the degree of correlation between sample size and number of unique haplotypes in each site revealed a negligible association between the two values ($r = 0.07$; $r^2 = 0.49\%$).

Total sample analyses

Comparisons with other species belonging to the genus *Clethrionomys* and with *C. glareolus* from different regions

revealed polymorphisms apparently unique to *Clethrionomys glareolus* from northern Ukraine (Table 1). Three polymorphisms, at positions 40, 157, and 170, were detected in our previous studies (for detailed analyses, see Wickliffe et al. [25]); however, an additional polymorphism was detected in our newest samples. An A↔G transition at position 143 is present in haplotype 14 and is restricted to the Paryshev locality. Whereas haplotypes 12, 13, and 15 contain combinations of nucleotides that are unique, the individual polymorphic sites within the remaining haplotypes are typical of either other populations of *C. glareolus* or other species of *Clethrionomys*. Haplotype 12 contains polymorphisms at positions 170 and 267. The variant at position 170 (C↔T transition site) is present in haplotypes 2 and 6 from contaminated sites and in *C. californicus*, *C. gapperi*, *C. rufocanus*, *C. rutilus*, and *C. glareolus* from areas outside of northern Ukraine. The variant at position 267 (C↔T transition site) is present in haplotypes 3 and 15, in haplotypes of *C. gapperi* and *C. rufocanus*, and in haplotypes of *C. glareolus* from areas outside of northern Ukraine. Haplotype 13 contains polymorphisms at positions 156 and 157 (C↔T transition sites). The variant at position 156 also is present in *C. gapperi* and *C. rutilus* haplotypes, and the variant at position 157 is present in haplotypes 2, 4, 7, 9, and 11 from Ukrainian samples. Additionally, *C. californicus*, *C. rufocanus*, *C. rutilus*, and non-Ukrainian *C. glareolus* haplotypes contain C↔T transitions at position 157. Haplotype 14 contains three variant positions (at sites 143, 152, and 157). Whereas the A↔G transition at position 143 is unique to the Paryshev samples, the C↔T transition site at position 152 also is found in haplotype 8 and in *C. gapperi*. The variant at position 157 is shared among several haplotypes and species, as noted above. Haplotype 15 varies at positions 56, 157, 170, and 267 and is the only haplotype within the northern Ukraine samples to vary at four nucleotide positions.

Table 3. Sample size (n), haplotype diversity estimates (h), and nucleotide diversity estimates (ND), with standard error (SE) included for diversity estimates, in sampled sites across all sampling periods^a

Site	n	h	SE	ND	SE
Chista	17	0.62	0.11	0.0030	0.0025
Nedanchichy	28	0.20	0.10	0.0015	0.0015
Oranoe 98	43	0.62	0.04	0.0025	0.0021
Oranoe 99	22	0.63	0.06	0.0027	0.0023
Glyboke	28	0.68	0.07	0.0042	0.0031
Red Forest 97	105	0.72	0.02	0.0047	0.0033
Red Forest S 98	8	0.82	0.10	0.0059	0.0044
Red Forest F 98	24	0.74	0.05	0.0039	0.0029
Red Forest 99	40	0.75	0.06	0.0051	0.0036
Red Forest 00	15	0.79	0.08	0.0061	0.0042
Red Forest 01	54	0.67	0.04	0.0059	0.0039
Krasnoye	13	0.69	0.12	0.0051	0.0037
Paryshev	49	0.59	0.07	0.0035	0.0027
Stupnikovo	22	0.68	0.07	0.0028	0.0024

^a Red Forest and Glyboke Lake are contaminated sites located near Pripjat, Ukraine. Chista, Nedanchichy, and Oranoe are reference sites used in previous studies. Krasnoye, Paryshev, and Stupnikovo are reference sites added in the present study based on proximity to contaminated sites. S = summer sampling period; F = fall sampling period.

The variant at position 56 (C \leftrightarrow T transition) also is present in haplotypes 5 and 9, in addition to *C. gapperi*, *C. rufocanus*, and non-Ukrainian *C. glareolus*. Nucleotide variants at position 157, 170, and 267 are present in other haplotypes, as discussed above.

Diversity estimates

Haplotype diversity (h) and nucleotide diversity (ND) estimates are summarized for the present and previous studies in Table 3. Haplotype diversity estimates are 0.69 ± 0.12 , 0.59 ± 0.07 , and 0.68 ± 0.07 for Krasnoye, Paryshev, and Stupnikovo, respectively. The estimate of h for Paryshev is similar to estimates for other reference localities (excluding Nedanchichy), but estimates of h for both Krasnoye and Stupnikovo are equal to or greater than diversity estimates for Glyboke Lake and for the last sampling period in the Red Forest. The standard error was used to evaluate differences in h between localities. Estimates of haplotype diversity at Krasnoye, Paryshev, and Stupnikovo do not appear to be significantly different from those of contaminated or other reference localities, except for Nedanchichy (Fig. 2). Nucleotide diversity estimates are 0.0051 ± 0.0037 , 0.0035 ± 0.0026 , and 0.0028 ± 0.0024 for Krasnoye, Paryshev, and Stupnikovo, respectively. Nucleotide diversity for Krasnoye is elevated compared to other reference sites and Glyboke Lake but is less than the average ND estimate for Red Forest. Nucleotide diversity for Paryshev is elevated as compared to other reference sites but is less than ND estimates for both Red Forest and Glyboke Lake. The ND estimate for Stupnikovo is equivalent to the average ND estimate for other reference sites if Nedanchichy is excluded from the average. As with estimates of haplotype diversity, estimates of ND for Krasnoye, Paryshev, and Stupnikovo do not appear to be significantly different from those of contaminated or reference localities except for the Nedanchichy reference locality (Fig. 3). Diversity values (h and ND) averaged across sampling periods are elevated in the Red Forest with respect to all other localities, but because some degree of temporal variation exists in these estimates, the latest sam-

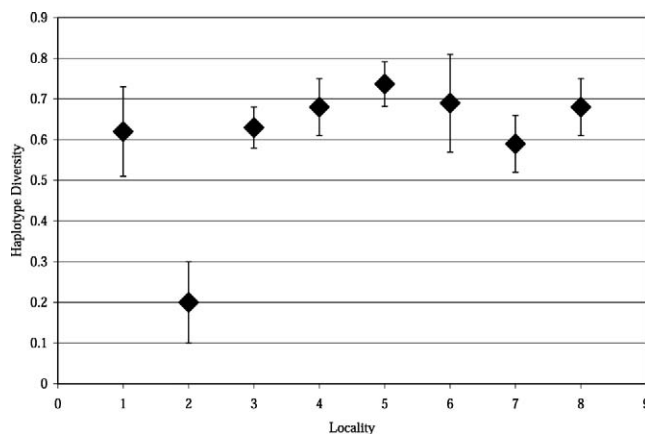


Fig. 2. Haplotype diversity estimates (h) for contaminated sites (5 = Red Forest and 4 = Glyboke Lake), relatively distance reference sites (1 = Chista, 2 = Nedanchichy, and 3 = Oranoe), and neighboring reference sites (6 = Krasnoye, 7 = Paryshev, and 8 = Stupnikovo) in Chernobyl, Ukraine. Standard error estimates are represented by vertical lines.

pling period is used as the basis for comparison in the above analyses.

Analysis of molecular variance

Detailed AMOVA results are shown in Table 4. When contaminated versus uncontaminated sites were evaluated, the majority of the variation, 84.23%, was partitioned within populations. Low variation among groups (0.15%; i.e., between contaminated and uncontaminated sites) does not account for a significant proportion ($p > 0.05$) of the total variation in the study populations. The remainder of the variation, 15.63%, is partitioned among populations within groups. Subsequent AMOVA analyses, in which populations were randomly assigned to groups, resulted in an increased amount of variation partitioned among groups; in each of these analyses, among-group variation accounted for a significant proportion of the total variation (data not shown).

Minimum evolution analysis

Minimum evolution analysis is compatible with the conclusions drawn by Wickliffe et al. [25]. Haplotype 2, which

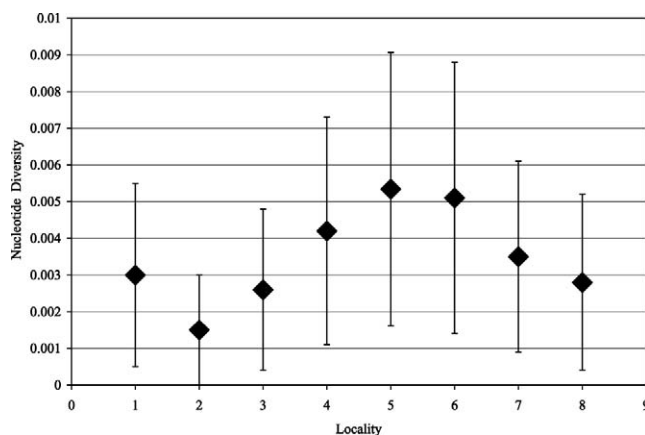


Fig. 3. Nucleotide diversity estimates for contaminated sites (Red Forest and Glyboke Lake), relatively distant reference sites (Chista, Nedanchichy, and Oranoe), and neighboring reference sites (Krasnoye, Paryshev, and Stupnikovo) in Chernobyl, Ukraine. Estimates of standard error are represented by vertical lines. Locality numbers are as given in Figure 2 caption.

samples from uncontaminated areas. Of specific interest was the Red Forest locality, in which higher levels of both haplotype diversity and nucleotide diversity were consistent across multiple sampling periods (1995–2001). Whereas aspects of the data from these studies suggested that genetic diversity in contaminated sites was correlated with radiation exposure, other evidence was in opposition to such a hypothesis. Consequently, authors in each of the studies concluded that patterns of diversity in contaminated regions could not be inextricably linked to the effects of radiation. The experimental design in the aforementioned study series, however, did not provide for specific assessment of ecological or demographic factors, particularly those related to geographic isolation, which may have contributed to observed differences between exposed and unexposed groups. Reference sites selected for the studies are, at minimum, 30 km from one another and from contaminated sites, and contaminated sites are separated by the Pripjat River system, representing a prospective barrier to gene flow. Stacy et al. [35] demonstrated the correlation between distance and genetic identity in bank voles, such that vole populations separated by distances greater than 8.5 km were less genetically similar to one another than were more proximal populations. Aars et al. [36] showed that linear distances of 2 km or greater were sufficient to restrict mitochondrial gene flow among vole populations. Consideration of these data prompted reevaluation of experimental design in the ongoing Chernobyl project, so the contribution of geographic isolation to differences among the populations could be assessed.

For the present study, mitochondrial sequence data from an additional 84 animals inhabiting the reference areas Krasnoye, Paryshev, and Stupnikovo were incorporated into the database ($n = 384$) established during previous studies conducted in the Chernobyl region. Selection criteria for the new reference sites included minimal radioactive contamination from the Chernobyl accident, proximity to highly contaminated areas, and absence of obvious physical barriers to dispersal between the reference sites and nearby contaminated sites (Fig. 1). If the variation observed in contaminated regions [23–25] is related primarily to geographic isolation of sites, then reference groups closely situated to contaminated sites would be expected to exhibit similar levels of diversity (h and ND). Alternatively, if diversity in contaminated sites is a result of exposure to chronic radiation, then highly contaminated sites should have the highest h and ND among sampled sites, and differences between contaminated and uncontaminated sites should be detectable even in adjacent reference localities. As described below, comparisons of genetic variation in exposed and neighboring unexposed groups revealed different patterns in h and ND but, overall, suggested that levels of diversity in contaminated regions are explained more plausibly by ecological and historical factors than by increased mutational pressure resulting from exposure to Chernobyl radiation.

Estimates of h in areas proximal to radioactively contaminated regions are similar to estimates for the most recently collected samples from contaminated sites (Table 3). These results are compatible with the hypothesis that variation in vole populations of northern Ukraine is produced by natural population processes rather than by an elevated mutation rate in contaminated sites. By contrast, h in Paryshev is lower than in most other areas, although the Nedanchichy reference locality continues to exhibit the lowest values for mtDNA diversity among the sampled sites. The disparate level of diversity in Paryshev may be partly attributable to distance from

other sites. Whereas Krasnoye and Stupnikovo are located within 6 km of Glyboke Lake and the Red Forest, respectively, Paryshev is separated from contaminated sites by greater distances (>30 km). As indicated above, genetic identity in bank voles is strongly correlated with distance; thus, it is not unexpected that Paryshev would exhibit less similarity to contaminated sites than more proximal reference sites.

Nucleotide diversity estimates reveal a different pattern than was evident in the mtDNA haplotypes. Nucleotide diversity is higher for Krasnoye than for other sampled sites, excluding the Red Forest, whereas ND for Stupnikovo and Paryshev is more reflective of estimates for outlying reference regions. Wickliffe et al. [25] suggested that ND could be the more sensitive of the two diversity measures (h and ND) in detecting increased mutation pressure. A conclusive statement regarding the significance of values or the pertinence of one type of estimate as opposed to the other, however, cannot be made without an accepted statistical method by which to assess differences in h and ND among sites.

On average, both h and ND for the Red Forest are the highest among sampled sites; however, the Red Forest data should be cautiously interpreted, given the substantial (albeit not statistically significant) fluctuations in diversity estimates over sampling periods at the site (Table 3). The number of samples collected and the number of sampling periods further complicates direct comparisons between the Red Forest and other sites. Other localities included in the present study typically were sampled only once, and in all cases, sample sizes are markedly lower than those for the Red Forest. Annual fluctuations in size and diversity of populations are well-documented phenomena in the bank vole. Thus, a paucity of samples from other collection areas, as well as a lack of more recent samples from the Red Forest, may be contributing to apparent differences among sites.

Analysis of haplotype distribution provides another means by which to assess variation in Ukrainian vole populations. Four unique haplotypes (i.e., haplotypes 12–15) and one haplotype previously associated only with contaminated regions were identified in samples from reference collections added in 2004, although four haplotypes identified in earlier studies as being unique to either contaminated or reference sites were not detected in these samples (Table 2). Additionally, a unique polymorphism at position 143 was detected in haplotype 14 that has not, to date, been observed in other *Clethrionomys* haplotypes (Table 1). The unique polymorphic site, as well as three of the four newly identified haplotypes, was detected only in Paryshev samples. The total number of haplotypes and the number of unique haplotypes is greater in Paryshev than in any other sampled site, including contaminated sites, and the composition of haplotypes at that location is distinct from other localities (Table 2). Sampling error is inadequate to explain all the observed differences because Paryshev is atypical even in comparison to areas in which relatively large numbers of samples have been collected (e.g., Red Forest). Additionally, whereas the correlation coefficient value indicates a moderate degree of correlation ($r^2 = 18\%$) between sample size and total number of haplotypes detected in each site, it indicates virtually no correlation ($r^2 = 0.49\%$) between sample size and number of unique haplotypes detected in each site. The apparent incongruity of the Paryshev group underscores the broad range of variability in bank vole populations and suggests that natural processes rather than radiation exposure

are the most significant moderators of diversity in Chernobyl residents.

Our results do not provide support for the hypothesis that exposure to low-dose, chronic radiation is the principal source of variation in Chernobyl populations. If chronic radiation were the primary instigator of genetic variability in Ukrainian vole populations, then we would expect to see clear differences between exposed and unexposed groups. On the contrary, no obvious demarcation is found between the groups, as evidenced by both phylogram and AMOVA analyses (Fig. 4 and Table 4), indicating that no singular process is responsible for producing the levels of genetic diversity documented in radioactively contaminated sites. Diversity estimates for both contaminated and neighboring uncontaminated regions are similar; furthermore, a negligible amount of the variation in these sites is attributable to differences between exposed and unexposed groups (Table 4). Our data offer little indication of a causal association between chronic exposure to Chernobyl radiation and genetic structure in resident populations and, thus, support the results of earlier studies [23–25]. Geographic isolation appears to have played a significant role in shaping these populations, although other demographic, ecological, and historical factors may have contributed to observed differences among sampled localities. Studies have linked a number of processes, including ancient colonization patterns [37,38], current levels of gene flow [39], and intermittent population bottlenecks [40], to patterns of diversity in rodent populations. For example, Bickham et al. [41] and Mendez-Harclerode et al. [39] documented levels of haplotype diversity as high as $h = 0.87$ and $h = 0.99$ in the Steller sea lion (*Eumetopias jubatus*) and the wood rat (*Neotoma micropus*), respectively. Because exposure to exogenous mutagens is not known to occur in the localities evaluated for these studies, high levels of diversity in the populations are thought to be related to ecological processes. Given that Chernobyl vole populations do not exhibit unusually elevated levels of diversity as compared to other mammalian populations, it seems reasonable to suggest that genetic diversity at Chernobyl is a function of normal population-level processes. Among the processes almost certainly influencing variability is repopulation of contaminated areas that experienced localized extinction as a result of the accident. Increased diversity in contaminated sites may be a corollary of metapopulation dynamics, whereby multiple source populations in close proximity to contaminated sites are contributing, or have recently contributed, to mtDNA heterogeneity in exposed populations (for additional discussion, see Matson et al. [23] and Baker et al. [24]). It also is possible that selective forces are decreasing levels of diversity in contaminated areas by eliminating animals with reduced fitness, resulting in the underestimation of mutation rates associated with radiation exposure. Ellegren et al. [42] and Møller and Mousseau [43] hypothesized that decreased reproductive fitness in Chernobyl barn swallows was linked to chronic radiation exposure; however, because the researchers did not provide information regarding dosimetry in the presumably exposed birds, the correlation between radiation exposure and reduced fitness is unsubstantiated. By contrast, other studies [4,5], in which levels of radioactivity were assessed for all collected specimens, found no significant effect on either species composition or population densities in small mammals from radioactive regions of Chernobyl. Their results suggest little to no reduction in population size associated with low-dose, chronic radiation exposure, thus casting doubt on the

notion that selection is reducing genetic diversity in contaminated areas.

Spatial variation in genetic and morphological characters is a universal characteristic in animal populations and has been documented in thousands of published accounts. Such variation is so prevalent that a study showing no appreciable spatial variation would be notable and surprising. Publications attempting to document the cause and effect of environmental contaminants on genetic and morphological variation must be held to higher standards than proximity of a point source, because organismal variation likely could be construed as being attributable to any real or artificially selected geographic point. Care should be taken to document radiation doses to the organisms sampled and to account equitably in experimental and control samples for potentially confounding factors to measured variation. The present study attempts to account for variations in spatial proximity and geographical features that may exacerbate variance among sites. Our results show that the underlying variability seen both within and among bank vole populations in the Chernobyl region is consistent with that expected from naturally occurring geographic variation. We conclude, therefore, that patterns of haplotype and nucleotide diversity in Chernobyl bank voles are not the result of increased mutation pressure related to low-dose, chronic radiation exposure.

Acknowledgement—We thank Ronald J. Kendall, Robert D. Bradley, and Sandra L. Diamond for their critique of this manuscript. We thank Hugo Mantilla-Meluk and Sergio S. Solari for assistance in creating the graphics and L. Rex McAliley for assistance with the analyses. We thank the Administration of the Chernobyl Exclusive Zone and the Ministry of Emergency Situations and Protection of the Population from the Consequences of the Chernobyl Nuclear Accident. We thank the personnel of the International Radioecology Laboratory in Slavutych and Chernobyl, Ukraine, particularly Mikhail Bondarkov, Sergey Gaschak, and Julia Goryanaya, for their help in data collection. This research has been supported by a contract between Texas Tech University and the U.S. Department of Energy (RWO-44) and the Biological Database Studies of Texas Tech University.

REFERENCES

1. Chesser RK, Sugg DW, Lomakin MD, Van Den Bussche RA, DeWoody JA, Jagoe CH, Dallas CE, Whicker FW, Smith MH, Gaschak SP, Chizhevsky IV, Lyabik VV, Buntova EG, Holloman K, Baker RJ. 2000. Concentrations and dose rate estimates of ^{134,137}Cesium and ⁹⁰Strontium in small mammals at Chernobyl, Ukraine. *Environ Toxicol Chem* 19:305–312.
2. Chesser RK, Rodgers BE, Wickliffe JK, Gaschak S, Chizhevsky I, Phillips CJ, Baker RJ. 2001. Accumulation of ¹³⁷Cesium and ⁹⁰Strontium from abiotic and biotic sources in rodents at Chernobyl, Ukraine. *Environ Toxicol Chem* 20:1927–1935.
3. Chesser RK, Bondarkov M, Baker RJ, Wickliffe JK, Rodgers BE. 2004. Reconstruction of radioactive plume characteristics along Chernobyl's western trace. *J Environ Radioact* 71:147–157.
4. Baker RJ, Hamilton MJ, Van Den Bussche RA, Wiggins LE, Sugg DW, Smith MH, Lomakin MD, Gaschak SP, Bundova EG, Rudenskaya GA, Chesser RK. 1996. Small mammals from the most radioactive sites near the Chernobyl nuclear power plant. *J Mammal* 77:155–170.
5. Baker RJ, Chesser RK. 2000. Letter to the editor: The Chernobyl nuclear disaster and subsequent creation of a wildlife preserve. *Environ Toxicol Chem* 19:1231–1232.
6. Fan YJ, Wang A, Sadamoto S, Ninomiya Y, Kotomura N, Kamiya K, Dohi K, Kominami R, Niwa O. 1995. Dose-response of a radiation induction of a germline mutation at a hypervariable mouse minisatellite locus. *Int J Radiat Biol* 68:177–183.
7. Fennelly J, Wright E, Plumb M. 1997. Mini- and microsatellite mutations in radiation-induced myeloid leukemia in the CBA/H mouse. *Leukemia* 11:807–810.
8. Barber R, Plumb MA, Boulton E, Roux I, Dubrova YE. 2002. Elevated mutation rates in the germ line of first- and second

- generation offspring of irradiated male mice. *Proc Natl Acad Sci U S A* 99:6877–6882.
9. Pogribny I, Raiche J, Slovack M, Kovalchuk O. 2004. Dose-dependence, sex- and tissue-specificity, and persistence of radiation-induced genomic DNA methylation changes. *Biochem Biophys Res Commun* 320:1253–1261.
 10. Shimada A, Eguchi H, Yoshinaga S, Shima A. 2005. Dose-rate effects on transgenerational mutation frequencies in spermatogonial stem cells of the Medaka fish. *Radiat Res* 163:112–114.
 11. Sugg DW, Bickham JW, Brooks JA, Lomakin MD, Jagoe CH, Dallas CE, Smith MH, Baker RJ, Chesser RK. 1996. DNA damage and radiocesium in channel catfish from Chernobyl. *Environ Toxicol Chem* 15:1057–1063.
 12. Rodgers BE, Wickliffe JK, Phillip CJ, Chesser RK, Baker RJ. 2001. Experimental exposure of naïve bank voles (*Clethrionomys glareolus*) to the Chernobyl, Ukraine, environment: A test of radioresistance. *Environ Toxicol Chem* 20:1936–1941.
 13. Weinberg HS, Korol AB, Kirzhner VM, Avivi A, Fahima T, Nevo E, Shapiro S, Rennert G, Platak O, Stepanova E, Skvarkaja E. 2001. Very high mutation rate in offspring of Chernobyl accident liquidators. *Proc R Soc Lond B Biol Sci* 268:1001–1005.
 14. Dubrova YE, Grant G, Chumak AA, Stezhka VA, Karakasian AN. 2002. Elevated minisatellite mutation rate in post-Chernobyl families from Ukraine. *Am J Hum Genet* 71:801–809.
 15. Möller AP, Mousseau TA. 2003. Mutation and sexual selection: A test using barn swallows from Chernobyl. *Evolution* 55:2097–2104.
 16. Wickliffe JK, Bickham AM, Rodgers BE, Chesser RK, Phillips CJ, Gaschak SP, Goryanaya JA, Chezhevsky I, Baker RJ. 2003. Exposure to chronic, low-dose-rate gamma-radiation at Chernobyl does not induce point mutations in Big Blue® mice. *Environ Mol Mutagen* 42:11–18.
 17. Dubrova YE, Plumb M, Brown J, Fennelly J, Bois P, Goodhead D, Jeffreys AJ. 1998. Stage specificity, dose response, and doubling dose for mouse minisatellite germ-line mutation induced by acute radiation. *Proc Natl Acad Sci U S A* 95:6251–6255.
 18. Kadhim MA, Marsden SJ, Malcomsen AM, Folkard M, Goodhead DT, Prise KM, Michael BD. 2001. Long-term genomic instability in human lymphocytes induced by single-particle irradiation. *Radiat Res* 155:122–126.
 19. Little JB. 2000. Radiation carcinogenesis. *Carcinogenesis* 21:397–404.
 20. Baker RJ, DeWoody JA, Wright AJ, Chesser RK. 1999. On the utility of heteroplasmy in genotoxicity studies: An example from Chernobyl. *Ecotoxicology* 8:301–309.
 21. Rodgers BE, Baker RJ. 2000. Frequencies of micronuclei in bank voles from zones of high radiation at Chernobyl, Ukraine. *Environ Toxicol Chem* 19:1644–1648.
 22. Wickliffe JK, Chesser RK, Rodgers BE, Baker RJ. 2002. Assessing the genotoxicity of chronic environmental irradiation by using mitochondrial DNA heteroplasmy in the bank vole (*Clethrionomys glareolus*) at Chernobyl, Ukraine. *Environ Toxicol Chem* 21:1249–1254.
 23. Matson CW, Rodgers BE, Chesser RK, Baker RJ. 2000. Genetic diversity of *Clethrionomys glareolus* populations from highly contaminated sites in the Chernobyl region, Ukraine. *Environ Toxicol Chem* 19:2130–2135.
 24. Baker RJ, Bickham AM, Bondarkov M, Gaschak SP, Matson CW, Rodgers BE, Wickliffe JK, Chesser RK. 2001. Consequences of polluted environments on population structure: The bank vole (*Clethrionomys glareolus*) at Chernobyl. *Ecotoxicology* 10:211–216.
 25. Wickliffe JK, Dunina-Barkovskaya YV, Gaschak SP, Rodgers BE, Chesser RK, Bondarkov M, Baker RJ. 2006. Variation in the mitochondrial DNA control region haplotypes in populations of the bank vole, *Clethrionomys glareolus*, living in the Chernobyl environment, Ukraine. *Environ Toxicol Chem* 25:503–508.
 26. Longmire JL, Maltbie M, Baker RJ. 1997. Use of “lysis buffer” in DNA isolation and its implication for museum collections. *Occasional Papers, The Museum, Texas Tech University* N 163: 1–4.
 27. Matson CW, Baker RJ. 2001. DNA sequence variation in the mitochondrial control region of red-backed voles (*Clethrionomys*). *Mol Biol Evol* 18:1494–1501.
 28. PE Applied Biosystems. 2001. *Sequencing Analysis Software*, Ver 3.4.1. Foster City, CA, USA.
 29. Oxford Molecular Group PLC. 1998. *AssemblyLIGN™ Software*, Ver 1.0.9. Oxford, UK.
 30. Sinauer Associates. 2002. *MacClade Software*, Ver 4.05. Sunderland, MA, USA.
 31. Genetics and Biometry Laboratory. 2000. *Arlequin Software*, Ver 2.000. Excoffier, Geneva, Switzerland.
 32. Nei M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York, NY, USA.
 33. Arizona State University. 2001. *Molecular Evolutionary Genetics Software (MEGA2)*. Tempe, AZ, USA.
 34. ESRI. 2005. *ESRI® ArcMap™ Software*, Ver 9.1. Redlands, CA, USA.
 35. Stacy JE, Jorder PE, Steen H, Ims RA, Purvis A, Jakobsen KS. 1997. Lack of concordance between mtDNA gene flow and population density fluctuations in the bank vole. *Mol Ecol* 6:751–759.
 36. Aars J, Ims RA, Liu HP, Mulvey M, Smith MH. 1998. Bank voles in linear habitats show restricted gene flow as revealed by mitochondrial DNA (mtDNA). *Mol Ecol* 7:1383–1389.
 37. Federov VB. 1999. Contrasting mitochondrial DNA diversity estimates in two sympatric genera of Arctic lemmings (*Dicrostonyx*: *Lemmus*) indicate different responses to Quaternary environmental fluctuations. *Proc R Soc Lond B Biol Sci* 266:621–626.
 38. Heckel G, Burri R, Fink S, Desmet JF, Excoffier L. 2005. Genetic structure and colonization processes in European populations of the common vole, *Microtus arvalis*. *Evolution* 59:2231–2242.
 39. Mendez-Harclerode FM, Hanson JD, Fullhorst CF, Milazzo ML, Ruthven DC, Bradley RD. 2005. Genetic diversity within the southern plains woodrat (*Neotoma micropus*) in southern Texas. *J Mammal* 86:180–190.
 40. Richman AD, Gerardo HL, Nash D. 2003. Evolution of MHC class II E-beta diversity within the genus *Peromyscus*. *Genetics* 164:289–297.
 41. Bickham JW, Loughlin TR, Calkins DG, Wickliffe JK, Patton JC. 1998. Genetic variability and population decline in Steller sea lions from the Gulf of Alaska. *J Mammal* 79:1390–1395.
 42. Ellegren H, Lindgren G, Primmer CR, Møller AP. 1997. Fitness loss and germline mutations in barn swallow breeding in Chernobyl. *Nature* 389:593–596.
 43. Møller AP, Mousseau TA. 2001. Albinism and phenotype of barn swallows *Hirundo rustica* from Chernobyl. *Evolution* 55:2097–2104.