

# ASSESSING THE GENOTOXICITY OF CHRONIC ENVIRONMENTAL IRRADIATION BY USING MITOCHONDRIAL DNA HETEROPLASMY IN THE BANK VOLE (*CLETHRIONOMYS GLAREOLUS*) AT CHORNOBYL, UKRAINE

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**Abstract**—This study was designed to investigate whether or not chronic exposure to Chernobyl radiation poses a molecular genetic risk to mammals by examining a relatively rapidly evolving genetic system, mitochondrial DNA (mtDNA). More mtDNA mutations (~19%) and an increase in mtDNA heteroplasmy (~5%) occurred in the cytochrome *b* gene of an exposed mother–embryo set when compared to a relatively unexposed mother–embryo set. However, this increase was not statistically significant ( $p > 0.05$ ). Our results, in conjunction with previous molecular genetic research on small mammals from Chernobyl, suggest that chronic exposure to environmental ionizing radiation does not increase the number of nucleotide substitutions, as predicted by studies using acute or subacute exposures. Thus, cumulative models of radiation risk would not appear to follow simple linear functions derived from high doses and dose rates. The equivocal nature of research regarding the effects of the Chernobyl accident indicates that future research is warranted such that models of chronic environmental exposure can be developed or refined. Although additional study is required to properly validate mtDNA heteroplasmy as a useful effect biomarker, examination of these data does not indicate that a significant risk to mtDNA exists in native rodents chronically exposed to both internal and external radiation.

**Keywords**—Mitochondrial DNA *Clethrionomys* Vole Radiation Chernobyl/Chornobyl

## INTRODUCTION

After the accident at the Chernobyl Nuclear Power Plant, Ukraine, in 1986, numerous studies have been conducted with the basic goal of documenting the biological impacts associated with environmental exposure to ionizing radiation [1–7]. Although research has demonstrated ill effects attributable to short-lived isotopes such as <sup>131</sup>iodine [4,7], data on the latent effects of long-lived isotopes such as <sup>137</sup>cesium and <sup>90</sup>strontium thus far are indeterminate. For example, research on repetitive DNA elements (i.e., mini- and microsatellites) in both plants and animals has suggested either an increased germline mutation rate [5,6,8,9] or an absence of an increased germline mutation rate [10]. Unfortunately, studies indicating genetic effects presumably resulting from radiation at Chernobyl have not documented individual exposure, rendering ecological and human health risk difficult to assess. Furthermore, studies investigating mini- and microsatellite mutation rates after exposure to ionizing radiation resulting from atomic bomb explosions at Hiroshima and Nagasaki, Japan, have not found increases in mutation rates [11,12]. This paradox indicates that a simple model of dose–response cannot be invoked in assessing risk from exposure to ionizing radiation or that experimental design must be robust to unknown sources of variation influencing conclusions drawn from studies.

Research effectively documenting individual exposure and dose with respect to the Chernobyl environment [13] has not found statistically significant evidence of clastogenic lesions, elevated mutation rates, elevated levels of mitochondrial DNA (mtDNA) heteroplasmy, or population-level effects in wildlife [10,14–16]. Therefore, resolving the potential mutagenicity of

the radioactive environment at Chernobyl remains a critical issue with respect to assessing the genetic risk to humans and wildlife.

This study was designed to assess genetic mutations resulting from chronic exposure to environmental radiation in a relatively rapidly evolving protein-coding gene. We chose to examine mutations in the mtDNA cytochrome *b* (cyt *b*) gene and estimate substitution frequencies and heteroplasmy (i.e., the presence of both wild and mutant mtDNA genotypes within an individual [17]) in the mtDNA. Mitochondrial DNA is an appropriate system in which to study genetic mutations because of its relatively high mutation rate [18]; its implication, including the cyt *b* gene [19,20], in a number of genetic disorders, including cancer [21–23]; and the multiplicity of the mtDNA within each cell [18,24]. The latter, in theory, allows the retention of mutant haplotypes below a threshold (upwards of 70% mutant [20]) at which oxidative phosphorylation is affected. In addition, the relative simplicity of the structure of the cyt *b* gene (a single exon of ~1,140 base pairs) and the well-characterized DNA sequence of the cyt *b* gene in numerous organisms make this gene particularly useful. Finally, the theoretical framework from which to assign the origin (i.e., germline or somatic) of mtDNA variants has been derived, allowing for estimation of both transgenerational and individual genetic risk [25].

An analytical approach recently has been developed that is designed to detect differences in the number of mtDNA substitutions and the level of heteroplasmy between individuals [14]. This approach was applied in a study investigating mtDNA heteroplasmy in a radioactively exposed female grassland vole (*Microtus arvalis*) and her embryos and an unexposed female and her embryos. A nonsignificant increase was found in the number of nucleotide substitutions in the cyt *b*

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gene of the exposed individuals. In a comparative study of internal exposure incurred by small mammal species living in the Chernobyl environment, Chesser and coworkers [13] determined that the bank vole (*Clethrionomys glareolus*) received a substantially higher internal, chronic dose than did *M. arvalis*. Bank voles inhabiting the most radioactive areas experience an intramuscular radiocesium activity and dose rate up to 164,952.8 Bq/g and 119.8 mGy/d, respectively, and an external dose rate of 54 mGy/d [13]. This is considerably higher than the maximum observed internal radiocesium activity and dose rate documented in *M. arvalis* of 2,626.2 Bq/g of muscle tissue and 1.9 mGy/d, respectively [13]. We make the assumption that external exposure and dose rate are approximately equal among species because both are collected from the same location [1]. The average activity (16,482.9 Bq/g) and dose rate (12.0 mGy/d) exhibited by *C. glareolus* inhabiting the Chernobyl region are well above current regulatory limits (1 mGy/d) set for human and wildlife exposure [26]. If one assumes a cumulative, linear model relating dose and effect, as do the existing regulations, the annual dose experienced by these voles far exceeds that documented to cause lethality in this species, laboratory mice (*Mus*), and humans [26]. Finally, the bank vole is well studied and is distributed throughout Europe and western Asia and as such may be useful as a sentinel in future environmental studies [27–29]. Therefore, we conclude that the bank vole is an appropriate model system in which to investigate mutation frequencies by using the mtDNA heteroplasmy approach. Our experimental approach is designed to test if a significant increase occurs in the frequency of mtDNA nucleotide substitutions and heteroplasmy between the experimental (exposed) and the reference (relatively unexposed) mother–embryo set.

## MATERIALS AND METHODS

### Experimental design

This study follows the experimental design, including the use of liver tissue as the source of DNA, and terminology of Baker et al. [14], with the exceptions detailed below. These authors [14] reported nucleotide site substitutions and mtDNA heteroplasmy in another arvicoline species, *Microtus arvalis*, by examining *cyt b* gene clones generated by the polymerase chain reaction. They analyzed one exposed mother–embryo set ( $n = 6$ ), one reference mother–embryo set ( $n = 6$ ), and a technical set ( $n = 6$ ) to estimate procedural error. This study differs in terminology and the model system selected for investigating mtDNA heteroplasmy in the following manner. First, individuals ( $n = 7$ ) in the exposed mother–embryo set are grouped and termed experimental series, individuals ( $n = 7$ ) in the unexposed mother–embryo set are grouped and termed reference series, and unique clones ( $n = 7$ ) used to estimate procedural error are grouped and termed technical series. Second, experimental and reference mother–embryo sets were specimens of the bank vole rather than *M. arvalis*. The experimental female (Texas Tech University [TTU]/Natural Science Research Laboratory [NSRL]-TK74138) was captured alive in the radioactively contaminated Red Forest approximately 1.5 km west of the Chernobyl Nuclear Power Plant (Universal Transverse Mercator [UTM] 36295545 Northing [N]5697040). This female's intramuscular radiocesium activity was estimated to be 73,083.3 Bq/g with an estimated dose rate of 53.1 mGy/d [13]. The reference female *C. glareolus* (TTU/NSRL-TK74342) was captured alive in a relatively uncontaminated area known as The Shop (UTM

36297452 N5663689). This female's intramuscular radiocesium activity was estimated to be 427 Bq/g with an estimated dose rate of 0.3 mGy/d [13]. Third, each female had 6 embryos instead of 5 as in Baker et al. [14], and all 12 (6 from each female) were used in these analyses. Fourth, 20 molecules/individual, homologous to the *cyt b* gene in the study by Baker et al. [14], were screened for site heteroplasmy instead of 10 molecules/individual *M. arvalis*. All polymerase chain reaction, cloning, and sequencing reactions follow Baker et al. [14]. All putative mutations were verified on both DNA strands.

### Genome assignment

A phylogenetic approach initially was used to determine if cloned molecules appeared to originate in the mitochondrial or nuclear genome (i.e., behaved as pseudogenes). Mitochondrial pseudogenes including *cyt b* pseudogenes presumably translocated to the nuclear DNA have been documented [30,31]. Mitochondrial DNA pseudogenes serve to confound analyses relying on estimating nucleotide substitutions in actual mtDNA gene copies because pseudogenes presumably have lost electron transport function and are no longer subject to the evolutionary constraints required of the functional gene. Furthermore, nucleotide substitutions in pseudogenes are regulated by nuclear DNA replication and repair processes. To estimate the phylogenetic affiliations among cloned molecules within an individual, we used the individual's direct (i.e., wild-type) *cyt b* DNA sequence, two additional *C. glareolus* (one from Finland and one from Wales), several additional species of *Clethrionomys* (*C. gapperi*, *C. rutilus*, and *C. rufocanus*), species within the closely related genus *Eothenomys*, and two species of *Microtus* (DNA sequences of species of *Clethrionomys* were provided by J. Cook [University of Alaska, Fairbanks, AK, USA]); species of *Eothenomys* and *Microtus* were obtained from GenBank [National Center for Biotechnology Information; Bethesda, MD, USA]). A cloned molecule falling outside of the monophyletic *C. glareolus* clade (monophyly reflected in parsimony, maximum-likelihood, and distance-based analyses) was suspected of being nuclear in origin and was removed from subsequent analyses.

### Statistical procedure, significance, and genetic endpoints

Both parametric (PROC FREQ; likelihood ratio chi-square [ $\chi^2$ ] for frequency data) and nonparametric (PROC NPAR1WAY; Kruskal–Wallis test [ $\chi^2$  approximation]) statistical procedures were used to test for significant differences between experimental, reference, and technical groups by using the SAS® version 6.03 program [32]. A one-tailed statistical approach was used because significant increases in the frequency of mutations after exposure to radiation are well documented, whereas significant decreases in the frequency of mutations (e.g., adaptive response) have been documented in few studies limited to the analysis of cell populations in vitro. We estimated genetic endpoints examined in the study of Baker et al. [14], including the number of unique variants (nucleotide substitutions), transition and transversion substitutions, and multiple substitutions per clone. In addition, we estimated the proportion of heteroplasmy, nucleotide insertions and deletions, nucleotide diversity, codon position bias, and mutational bias in each series. Nucleotide diversity is the probability that any two randomly chosen homologous nucleotides are different [33]. Proportion of heteroplasmy is simply defined as the percentage of clones with a mutation. The exact nature of each

Table 1. Number of mitochondrial DNA cytochrome *b* (*cyt b*) nucleotide site substitutions, transition/transversion substitution ratio (TS/TV), and the percentage of clones observed to have multiple substitutions in each series. The experimental series is represented by one exposed mother–embryo set ( $n = 7$ ) collected from the radioactively contaminated Red Forest region near Chernobyl, Ukraine (UTM 36295545 U5697040). The reference series is represented by one unexposed mother–embryo set ( $n = 7$ ) collected from the relatively uncontaminated Shop region near Chernobyl (UTM 36297452 U5663689). The technical series is represented by unique *cyt b* clones ( $n = 7$ ) used for estimating procedural error. Values in parentheses are taken from Baker et al. [14] and presented for comparison

Series	Site substitutions <sup>a</sup>	TS/TV <sup>b</sup>	% with multiple substitutions (% clones with two or more)
Experimental ( $n = 7$ )	138 (15)	100/25 (11/4)	22.3 (0.03)
Reference ( $n = 7$ )	112 (10)	90/19 (10/0)	22.0 (0)
Technical ( $n = 7$ )	71 (6)	52/14 (6/0)	13.7 (0)

<sup>a</sup> Includes insertion/deletion mutations.

<sup>b</sup> Does not include insertion/deletion mutations.

nucleotide substitution was characterized in each series. For example, mutations were further subdivided from nonspecific transitions and transversions into specific A  $\rightarrow$  G transitions and A  $\rightarrow$  T or A  $\rightarrow$  C transversions. This was done for each substitution to determine if any possible mutational bias existed in the three series. Statistical significance was assessed with an alpha level of  $p < 0.05$ .

## RESULTS

### Frequency of mutations, transitions and transversions, and multiple mutations

Body burden and dose rate estimates for the two female bank voles used in this study indicate that the exposed female encountered significantly more radioactivity than did the reference female. The exposed female's intramuscular radiocesium activity was approximately 170 times that of the reference female. Eight *cyt b* clones (four from the experimental series, four from the reference series, and none from the technical series) were removed before mutation analysis because they failed to meet our criterion regarding functional gene copies. One hundred thirty-nine clones were used from the experimental series, 132 clones were used from the reference series, and 134 clones were used from the technical series to generate the molecular estimates. To facilitate comparison with the study of Baker et al. [14], values observed in this study for the endpoints estimated in their study are presented in Table 1. Approximately 19% more mutations were observed in the experimental series than in the reference series. However, this difference was not statistically significant ( $\chi^2 = 2.78$ ,  $p > 0.09$ ). Both experimental ( $\chi^2 = 6.56$ ,  $p < 0.02$ ) and reference ( $\chi^2 = 4.50$ ,  $p < 0.03$ ) series had significantly more mutations than the technical series. Both transition and transversion substitutions (Table 1) were observed in each series, in contrast to the transversion bias reported by Baker et al. [14]. The ratio of transition to transversion substitutions did not differ significantly between any of the three series (experimental vs reference [ $\chi^2 = 0.25$ ,  $p > 0.61$ ]; experimental vs technical [ $\chi^2 = 0.04$ ,  $p > 0.84$ ]; reference vs technical [ $\chi^2 = 0.38$ ,  $p > 0.53$ ]). The number of clones with multiple substitutions in the experimental (22.3%) and the reference (22.0%) series was essentially equal and not significantly different ( $\chi^2 = 1.43$ ,  $p > 0.83$ ). Both experimental ( $\chi^2 = 18.94$ ,  $p < 0.01$ ) and reference ( $\chi^2 = 11.93$ ,  $p < 0.02$ ) series had significantly more clones with multiple substitutions than the technical series (13.7%).

### Nucleotide diversity, proportion of heteroplasmy, and mutational spectra

Additional endpoints estimated in this study are presented in Table 2 and Figure 1. Nucleotide diversity was significantly higher in the experimental series (0.005;  $\chi^2 = 5.59$ ,  $p < 0.02$ ) in comparison to the technical series (0.003). Nucleotide diversity was not significantly elevated in comparisons between the experimental and reference series (0.004;  $\chi^2 = 1.80$ ,  $p > 0.17$ ) or between the reference and technical series ( $\chi^2 = 3.43$ ,  $p > 0.06$ ). The proportion of heteroplasmy was not significantly different between the experimental (59.7%) and reference (54.5%) series ( $\chi^2 = 1.36$ ,  $p > 0.24$ ). However, both experimental ( $\chi^2 = 7.65$ ,  $p < 0.01$ ) and reference ( $\chi^2 = 6.63$ ,  $p < 0.02$ ) series had a significantly higher proportion of heteroplasmy than did the technical series (32.8%). No significant differences were found between the three series with respect to mutational bias (Fig. 1; experimental vs reference [ $\chi^2 = 7.60$ ,  $p < 0.57$ ]; experimental vs technical [ $\chi^2 = 5.26$ ,  $p > 0.72$ ]; reference vs technical [ $\chi^2 = 3.56$ ,  $p > 0.89$ ]).

### DNA sequence deposition

The complete *cyt b* gene sequence for each adult bank vole was deposited (accession numbers AF318584-TK74138 and AF318585-TK74342) in GenBank (National Center for Biotechnology Information, Bethesda, MD, USA). This sequence represents that generated with the direct polymerase chain reaction and cycle sequencing procedure outlined by Baker et al. [14] and served as the wild-type sequence representing the

Table 2. Nucleotide diversity, the number of nucleotide substitutions across codon position, and the proportion of heteroplasmy observed in each series. The experimental series is represented by one exposed mother–embryo set ( $n = 7$ ) collected from the radioactively contaminated Red Forest region near Chernobyl, Ukraine (UTM 36295545 U5697040). The reference series is represented by one relatively unexposed mother–embryo set ( $n = 7$ ) collected from the relatively uncontaminated Shop region near Chernobyl (UTM 36297452 U5663689). The technical series is represented by unique cytochrome *b* clones ( $n = 7$ ) used for estimating procedural error

Series	Nucleotide diversity	Codon position (1:2:3)	Proportion of heteroplasmy (%)
Experimental ( $n = 7$ )	0.005	47:34:44	59.7
Reference ( $n = 7$ )	0.004	47:27:35	54.5
Technical ( $n = 7$ )	0.003	29:15:22	32.8

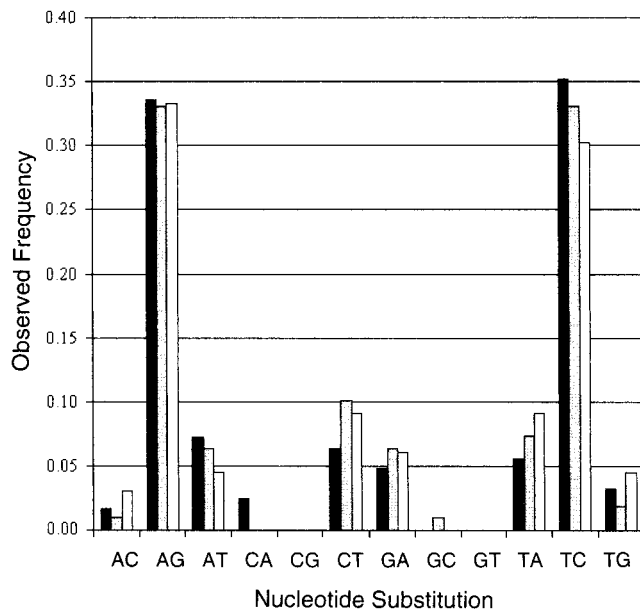


Fig. 1. Mutational spectra for experimental (black), reference (gray), and technical series (white). Nucleotide pairs along the abscissa represent the presumed wild-type nucleotide followed by the mutant nucleotide. Wild-type status was assigned by the presence, in each specimen's DNA sequence, derived by direct sequencing of the amplified gene. This portion of the cytochrome *b* gene has an inherent bias towards adenine (A, 30.5%), cytosine (C, 28.3%), and thymine (T, 28.5%) (percentages were derived from the direct sequences of the bank vole). AC implies an A to C transversion in which A represents the wild-type state and C represents the mutant state.

standard for all mutation and heteroplasmic comparisons. The *cyt b* sequence for the two adult bank voles differed by a cytosine–thymine (C–T, TK74138–TK74342) transition at position 592 of the gene (codon position 1), a thymine–adenine (T–A) transversion at position 915 (codon position 3), and a guanine–adenine (G–A) transition at position 963 (codon position 3). No direction is implied by the presentation of the DNA sequence differences, and the predicted amino acid sequences from both of the DNA sequences are identical. All embryos shared their respective dam's *cyt b* gene sequence.

## DISCUSSION

### *Rationale for investigating protein-coding DNA mutations*

Genetic mutations resulting from exposure to ionizing radiation are believed to lead to an increased risk of cancer and mortality [34]. If mutations in the genome impact organisms in the manner suggested by these effects, then one primary means would be by altering the DNA base pair sequence in protein-coding regions of genes. The majority of genetic research concerning environmental radiation has focused on chromosomal aberrations, DNA mutations in heterochromatin, or mini- and microsatellites that are not located in protein-coding DNA regions [5,6,9,10,16,35]. Comparatively few studies have investigated the impact of environmental radiation on protein-coding DNA sequences [14,36]. In fact, most investigations of nucleotide substitutions in protein-coding DNA after exposure to environmental mutagens, including nonradioactive contaminants, have focused on specific substitutions in cancerous tissues [37,38]. This research is valuable from a molecular mechanistic standpoint. However, in the absence of cancerous lesions, as is the case with the rodents examined from the Chernobyl region, direct comparisons between stud-

ies are difficult. A need exists to further develop genetic biomarkers after exposure to environmental mutagens, including the detection of point mutations in protein-coding loci [39]. Investigation of mutation rates and frequencies in both germline and somatic tissues when organisms are exposed to environmental mutagens should include protein-coding genes [40]. However, problems arise in the technical aspects used to resolve each point mutation and in documenting statistically relevant changes in the mutation rate of protein-coding genes [41,42].

### *Protein-coding gene mutations and their detection*

Studies on protein-coding genes often do not directly examine the entire DNA sequence of the gene but rely instead on other methods. The largest study examining nonclinical radiation exposure in humans relied on allozyme loci and variations in electrophoretic mobility to discern effects on protein-coding genes [43]. However, electrophoretic mobility may not differentiate all nucleotide sequence variants [44]. Other methods used to detect point mutations resulting from xenobiotic insult include chemical or enzymatic cleavage of nucleic acid hybrids, *Salmonella* testers, and *lacI* transgene expression. The first two methods often are used to identify specific nucleotide mismatches, whereas the latter may be used to identify certain types of mutations (i.e., missense or nonsense mutations) [42,45,46]. These approaches allow for rapid quantification of certain nucleotide substitutions in DNA sequences, especially in heterozygote conditions, but are not sensitive to all nucleotide substitutions (e.g., those mutations that are at or near the ends of amplified genes, or those mutations that do not change the amino acid sequence of the gene) or conditions where the proportion of mutant gene copies falls below 30% of the wild-type gene copy. In addition, mutagenic investigations of transgenes, although valuable from a strict mutagenesis perspective, target foreign genes not considered functionally important to the study organism.

We have employed a more sensitive approach to mutation detection. By using nucleotide sequencing, we can examine all nucleotide substitutions in a protein-coding DNA sequence, the mtDNA *cyt b* gene. This gene is present in multiple copies per cell and has a relatively high inherent rate of evolution when compared to other mitochondrial and nuclear protein-coding genes. Cytochrome *b* theoretically should provide a sensitive measure of increased mutation pressure resulting from chronic irradiation, if such a pressure exists. Although Baker et al. [2,3] retracted their original conclusions that the Chernobyl environment increased the mtDNA mutation rate in two species of *Microtus* by one to two orders of magnitude, they did observe an increase ( $p > 0.05$ ) in *cyt b* gene variation in the populations inhabiting the Chernobyl environment.

### *Radiation-induced *cyt b* gene mutations—Sensitivity to chronic irradiation?*

Ionizing radiation is well documented to cause genetic mutations, including nucleotide substitutions [47,48]. Also, the rodents inhabiting the Chernobyl region are well documented to be chronically exposed to high levels of environmental radiation [13]. Therefore, if ionizing radiation induces nucleotide substitutions in a dose-dependent manner, an alternative to the null hypothesis states that significantly more *cyt b* gene nucleotide substitutions will occur in clones from the experimental series in comparison to the reference series. When using *M. arvalis*, Baker and coworkers [14] found more *cyt b* mu-

tations in the exposed mother–embryo set, although this was not statistically significant ( $p > 0.05$ ). A power test of these data (results not shown) revealed that a doubling of the number of base pairs examined in the *Microtus* data set would have yielded a significantly elevated mutation frequency had the number of mutations observed remained constant. Therefore, we doubled the number of clones and hence base pairs examined. The other substantial change in our experimental design from that of Baker et al. [14] was that we chose a different rodent model system to increase the level of exposure to radiation by 2.3 times that of the study of Baker et al. [14]. We assumed that if *Clethrionomys* and *Microtus* experience the same risk of mutation per unit of absorbed energy or radioactivity then these two refinements increased the probability of detecting a significantly increased mutation frequency. This is especially pertinent considering the currently applied, although debated, linear, no-threshold dose–response model of genetic impacts resulting from exposure to ionizing irradiation (see Kirsch-Volders et al. [49] for a discussion of threshold concepts).

*Cytochrome b gene mutations and the Chernobyl environment: Interpretation, expectations, and dose–response relationship*

Although more nucleotide substitutions (26 total, or ~4 on average per individual examined) were found in the clones from the exposed mother–embryo set, this elevated frequency was not statistically significant. Clones from the experimental series had 19% more nucleotide substitutions than clones in the reference series and 94% more nucleotide substitutions than clones in the technical series. In contrast, clones from the biological experimental series in the *Microtus* data set had 50% more nucleotide substitutions than clones from the biological control series and 150% more nucleotide substitutions than clones from the technical control series [14]. The internal radiocesium activity in the experimental female *Clethrionomys* in this study was calculated to be 2.3 times that of the exposed female *Microtus* examined in the study of Baker et al. [14]. Therefore, either a simple dose–response relationship cannot be invoked with respect to mtDNA nucleotide substitutions in these two species, or the two species differ in their response to chronic exposure to ionizing radiation.

International standards regulating exposure to radiation assume a simple, linear, nonthreshold response to radiation and that dose is cumulative (e.g., acute and chronic exposure do not differ) and that rodent species are likely to respond in an equivalent fashion to ionizing radiation [26]. Examination of our results suggests that inherent differences exist between chronic, low-dose irradiation and acute irradiation. Differences also may exist among the sensitivity of various species to chronic irradiation, but these too serve to indicate that natural variation in response to chronic, low-dose irradiation must be assessed before invoking the current risk assessment model. Considerably more technical error (laboratory-induced substitutions) was observed in this study (1/709 for *C. glareolus*, 1/3,333 for *M. arvalis*), although in both data sets, technical error was significantly lower ( $p < 0.05$ ) than the biological mutation frequency in both experimental and reference (biological control) series. Thus, the approach developed by Baker and coworkers [14] reveals considerable biological signal in these two species of rodent. In both species, exposed mother–embryo sets exhibit more nucleotide substitutions (1/395 for *C. glareolus*, 1/1,852 for *M. arvalis*) in the *cyt b* gene than

the reference mother–embryo sets (1/469 for *C. glareolus*, 1/2,273 in *M. arvalis*). This trend may indicate an increased mutation pressure in the mtDNA genome of these two species resulting from the Chernobyl environment. However, this point lacks statistical support. As in the study of Baker et al. [14], a power test (0.99) of these data, assuming the observed trend continued, revealed that doubling the number of clones analyzed would result in a significantly ( $p < 0.05$ ) elevated level of *cyt b* gene heteroplasmy. However, additional empirical data (i.e., more clones and more mother–embryo sets) must be examined to confirm this technique, statistically validate this potential trend, and characterize the nature of increased heteroplasmy as a population phenomenon. Moreover, the role of DNA repair will need to be addressed because recent research indicates that mtDNA is governed by most known repair systems [23,50]. This includes base excision repair, which presumably is responsible for the repair of the nucleotide damage this study is designed to detect (i.e., misrepaired altered bases). The possibility exists that DNA damage is occurring in the *cyt b* gene of exposed animals, but repair capabilities simply are restoring the wild-type state. This has not yet been investigated. Finally, this approach requires laboratory validation to further its possible field application as an effect biomarker. A laboratory study, preferably on isogenic *Mus domesticus* matched in age class, in which radiation doses are tightly controlled, will allow for a robust analysis of dose–response relationships in the possible induction of mtDNA (*cyt b*) substitutions, insertions and deletions, and levels of heteroplasmy.

### Conclusion

Resolution to the question of “What are the genetic consequences associated with exposure to the Chernobyl environment?” remains unclear. The rodent model systems *M. arvalis* and *C. glareolus* suggest that no significant risk to the mtDNA *cyt b* gene exists in voles chronically exposed to high levels of environmental radiation. Additional research on these native model systems in conjunction with environmental studies conducted on mammalian model systems (e.g., *Mus musculus*) should help elucidate the extent to which the Chernobyl environment poses a significant wildlife and human genetic risk. These studies will assist in understanding what impact chronic environmental exposure to ionizing radiation, dose, and dose rate may have on genetic material.

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