

INTEGRATION OF GENOTOXICITY AND POPULATION GENETIC ANALYSES IN
KANGAROO RATS (*DIPDOMYS MERRIAM*) EXPOSED TO RADIONUCLIDE
CONTAMINATION AT THE NEVADA TEST SITE, USACHRISTOPHER W. THEODORAKIS,[†] JOHN W. BICKHAM,^{*†} TRIP LAMB,[‡]PHILIP A. MEDICA,[§] and T. BARRETT LYNE[†][†]Texas A&M University, Department of Wildlife and Fisheries Sciences, College Station, Texas 77843, USA[‡]East Carolina University, Department of Biology, Greenville, North Carolina 27858, USA[§]U.S. Geological Survey/Biological Resources Division, Las Vegas, Nevada 89108

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Abstract—We examined effects of radionuclide exposure at two atomic blast sites on kangaroo rats (*Dipodomys merriami*) at the Nevada Test Site, Nevada, USA, using genotoxicity and population genetic analyses. We assessed chromosome damage by micronucleus and flow cytometric assays and genetic variation by randomly amplified polymorphic DNA (RAPD) and mitochondrial DNA (mtDNA) analyses. The RAPD analysis showed no population structure, but mtDNA exhibited differentiation among and within populations. Genotoxicity effects were not observed when all individuals were analyzed. However, individuals with mtDNA haplotypes unique to the contaminated sites had greater chromosomal damage than contaminated-site individuals with haplotypes shared with reference sites. When interpopulation comparisons used individuals with unique haplotypes, one contaminated site had greater levels of chromosome damage than one or both of the reference sites. We hypothesize that shared-haplotype individuals are potential migrants and that unique-haplotype individuals are potential long-term residents. A parsimony approach was used to estimate the minimum number of migration events necessary to explain the haplotype distributions on a phylogenetic tree. The observed predominance of migration events into the contaminated sites supported our migration hypothesis. We conclude the atomic blast sites are ecological sinks and that immigration masks the genotoxic effects of radiation on the resident populations.

Keywords—Population genetics *Dipodomys merriami* Chromosomal damage Nuclear weapons Nevada Test Site

INTRODUCTION

Exposure to environmental contaminants can have various effects on natural populations. For example, acute or chronic toxic exposure can lead to genetic alterations at either the somatic (genotoxic) or the population genetic level. Although these two genetic effects may be correlated by a common etiology (contaminant exposure), their ultimate expression involves separate biological processes.

Somatic-level (genotoxic) effects are due to the direct interaction of contaminants with DNA. Chromosomal aberrations are often the result of such interactions and can be revealed by examination of whole-cell preparations for micronuclei [1] or by flow cytometry [2]. Micronuclei may form as a result of chromosomal fragmentation or interruptions in mitotic spindle formation and can be detected with standard cytological staining and microscopy [1]. Flow cytometry can be used to quantify chromosomal damage by analyzing cell-to-cell variation in DNA content (coefficient of variation [CV]). Higher CVs are indicative of increased chromosomal damage [2]. Recent studies have revealed higher CVs in vertebrates exposed to radionuclides and other mutagenic chemicals [3,4].

A second type of genetic effect may occur not through direct alteration of the DNA molecule itself but by decreased survival, reproduction, and/or longevity of individuals in affected populations (population genetic effects). Genetic bottlenecks or pollutant-induced selection can result, altering genetic diversity or allele frequencies and ultimately affecting the adapt-

ability, viability, and persistence of the population [5,6]. Moreover, selection by and adaptation to pollutants could complicate environmental risk assessments [7]. Conversely, the genetic structure of populations also may be affected by direct genotoxic exposure if the heritable mutation rate increases. Previous investigators have attempted to detect radiation-induced mutations and population genetic effects in rodent populations using mitochondrial DNA [8]. Other researchers [9] have developed a novel technique to investigate mutations in wild populations, which they have termed “terminal branch haplotype analysis.” This technique uses DNA sequence analysis and examination of haplotype phylogenies to discriminate between newly arisen DNA sequence variants (probable recent mutations) and historical polymorphism. Such an approach could prove useful in endeavors that use population genetic analysis as a toxicological tool.

Numerous studies have employed population genetics in toxicological investigations [10], the majority of which used allozyme techniques. However, the use of other genetic markers for such a purpose has received much less attention, and few comparative studies have examined both population genetic and somatic effects in the same species [11]. Such an integrative approach would be advantageous because changes in population genetic structure are not specifically indicative of contaminant exposure—as are genotoxic effects—so parallel responses of genotoxic effects may help distinguish natural versus anthropogenic etiologies of population genetic variation and because patterns of gene flow—an indication of dispersal—may aid interpretation of patterns of relative genotoxic response within and among populations. For example,

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extensive migration among contaminated and reference sites may increase within-site variation and decrease among-site variation, obfuscating patterns of contaminant response.

The U.S. Department of Energy's Nevada Test Site represents a unique area within the United States to examine the ecological effects of nuclear weapons. Between 1951 and 1963, a total of 105 atmospheric tests was conducted at the site or its associated bombing range. Herein, we examine clastogenic responses and population-genetic structure of a terrestrial vertebrate, Merriam's kangaroo rat (*Dipodomys merriami*), from contaminated ground-zero atomic blast sites where aboveground nuclear explosions were conducted as recently as 1957. *Dipodomys merriami* exhibits several behavioral and life history traits that make it suitable for this type of study. First, it has a relatively short generation time [12], and thus populations would be expected to respond relatively rapidly to a novel stressor, such as anthropogenic contamination. Second, as a burrowing species, it is likely to be exposed to any soil contamination. *Dipodomys merriami* is also an important component of desert ecosystems [13] and so merits consideration as a species of concern in environmental risk assessments. Finally, given their life history strategies, desert rodents are considered to be particularly vulnerable to the effects of contamination [14].

Two molecular markers were employed to examine the population genetic variation in this species: randomly amplified polymorphic DNA (RAPD) and mitochondrial DNA (mtDNA) control region markers. Previous RAPD surveys have revealed that radionuclide exposure altered genetic variability and population genetic structure of the western mosquitofish (*Gambusia affinis*) in Oak Ridge, Tennessee, USA, and eastern mosquitofish (*G. holbrooki*) from the Savannah River Site, South Carolina, USA [15]. The RAPD markers also correlated with relative fitness in contaminated habitats [15]. The mitochondrial control region was chosen as a second genetic marker because it is highly variable in mammalian populations [16], and previous studies have reported lower mitochondrial diversity for populations inhabiting contaminated versus reference areas [17].

The objectives of this study were to examine the effects of radionuclide contamination on kangaroo rat populations at the somatic (flow cytometry and micronucleus analyses) and population genetic levels (RAPD and mtDNA), to compare patterns of interpopulation differences at these two levels, and to use population genetics as an aid in interpreting patterns of genotoxic effects.

MATERIALS AND METHODS

Study site and sample collection

Individuals were captured in Sherman live traps from four sites at or near the U.S. Department of Energy's Nevada Test Site (Fig. 1). Two of these sites (ground-zero T1 and ground-zero T4, hereafter referred to as T1 and T4) were located in Yucca Flat, at the epicenter of aboveground nuclear weapons test detonations conducted on towers. Four tests were conducted at T1, including EASY in 1952 (12 kilotons [kt]), SIMON in 1953 (43 kt), APPLE II in 1955 (29 kt), and GALILEO in 1957 (11 kt). Four tests were conducted at T4, including FOX in 1952 (11 kt), NANCY in 1953 (24 kt), APPLE I in 1955 (14 kt), and KEPLER in 1957 (10 kt). Two reference sites (R1 and R2) were located approx. 50 km south of the tower sites (T1 and T4) between Mercury and Indian Springs, Nevada. Site R1 is located in Nye County, Nevada,

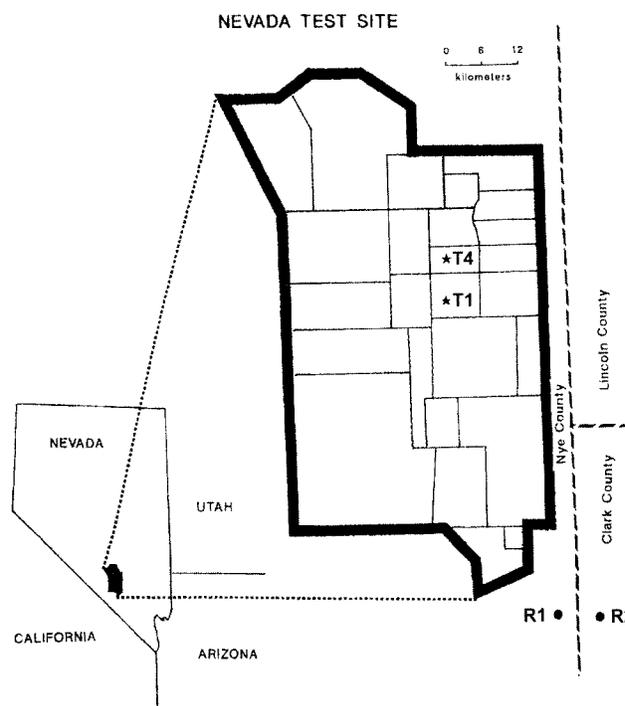


Fig. 1. Locations of four kangaroo rat populations in and adjacent to the U.S. Department of Energy's Nevada Test Site. Populations were sampled from two ground-zero atomic blast sites (T1 and T4) and two uncontaminated reference sites (R1 and R2).

at coordinates T 16S, R 54 E, sec 18; site R2 is in Clark County, Nevada, located at coordinates T 16S, R 54E, sec 22 (Fig. 1).

Animals were collected from April 1–16 and May 20–26, 1991. During the first collection period, animals were sacrificed on site and spleen samples frozen in liquid nitrogen. For the second collection period, animals were shipped live to Texas A&M University (College Station, TX, USA), where spleen tissues were removed and frozen in liquid nitrogen. Long-term storage of samples was in an ultracold freezer (-80°C). The sample sizes for populations R1, R2, T1, and T4 were, respectively, 24, 11, 15, and 28.

Radiation dosimetry

Radiation exposure measurements were recorded at each trapping location in the ground-zero locations; each consisted of three separate contact surface readings within 25 cm of the trap. Radiation measurements were recorded with a Ludlum 19 micro-R meter (Ludlum Measurements, Sweetwater, TX, USA).

Analysis of chromosomal damage

All individuals were analyzed for micronucleus and flow-cytometric analyses.

Micronucleus assay. Samples of blood were collected via vascular or cardiac puncture. Blood smears were stained with Wright Geimsa stain and scored visually using oil-immersion light microscopy. Micronucleus frequency was scored per 1,000 polychromatic erythrocytes examined. The Wilcoxon rank sum statistic was used to test differences among populations in terms of the average number of micronuclei per individual.

Flow cytometry. Spleen samples were analyzed via flow

cytometry according to previously described methods [4]. Nuclear suspensions, stained with propidium iodide, were analyzed with a Coulter Epics Profile II flow cytometer (Coulter Corporation, Hialeah, FL, USA). All samples were assayed on the same day, and samples were coded and randomized such that site and treatment were unknown during the assay. Because the data were not normally distributed, statistical differences between contaminated and reference populations were tested using the nonparametric Kruskal–Wallis test.

DNA extraction

The DNA was extracted for both RAPD and mtDNA analyses using approx. 10 to 50 mg of spleen tissue from each individual using previously described procedures [15].

RAPD analysis

Polymerase chain reaction for RAPD analysis was run on a Stratagene (La Jolla, CA, USA) Robocycler®, with denaturing, annealing, and elongation temperatures (and times) of 92° (60 s) 42° (30 s), and 72° (120 s) for two cycles and of 92° (20 s), 42° (30 s), and 72° (120 s) for 38 cycles. Primers were purchased from Operon Technologies (Alameda, CA, USA) (OPD primers) or from the Biotechnology Laboratory, Nucleic Acid and Protein Servicing Unit of the University of British Columbia (Vancouver, BC, Canada) (UBC primers). Sequences of RAPD primers are listed as follows: OPD2 GGA-CCCAACC, OPD7 TTGGCACGGG, OPD8 GTGTGCCCCA, OPD20 ACCCGGTCAC, UBC2 CCTGGGCTTG, UBC12 CCTGGGTCCA, and UBC16 GGTGGCGGGA. Magnesium, primer, and *Taq* polymerase concentrations were optimized for each primer to give the brightest, clearest banding patterns. The additional components of the reaction buffer consisted of 10 mM Tris, 50 mM KCl, and 1% Triton X-100 and 10 ng DNA in a total volume of 12.5 µl. Amplification products were separated electrophoretically in a 3% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. The presence or absence of bands was scored visually. All amplifications were performed in duplicate to identify non-reproducible bands, which were not included in the analysis. In order to minimize the possibility of misidentification of bands, only those bands that stained clearly and brightly were included in the analysis.

Similarity indices (*S*) were calculated between all possible pairs of individuals, according to the formula of Lynch [18] and as described in [15]. Genetic diversity was represented by the average similarity index between all pairs of individuals within each population (*S_w*). Genetic distance was calculated using the same formula, with paired individuals taken from separate populations (*S_b*). Variances of *S* (Var[*S*]) were computed according to Lynch [18]. Significant differences between similarity indices were tested using Student's *t* test: $(S_1 - S_2) / [\text{Var}(S_1) + \text{Var}(S_2)]^{1/2}$, with ∞ degrees of freedom.

Additional analysis of population genetic structure was performed as described in Lynch and Milligan [19], where only RAPD bands whose frequency was less than 0.97 were used. The statistics calculated using these techniques included *F_{st}* and average gene diversity (*H*). Differentiation among populations was determined by calculating *F_{st}* between all pairs of populations. Frequencies of RAPD bands were compared between contaminated and reference populations as the number of individuals displaying the band divided by the total number of individuals in the population.

Mitochondrial DNA analysis

Polymerase chain reaction amplification. A ≈1,000-bp segment containing the mitochondrial control region was amplified from total DNA samples using the following primers: LGL 283, 5' TACTACTGGTCTTGTAAC 3', LGL 282, 5' AAGGCTAGGACCAAACCT 3'. The reaction buffer consisted of 10 mM Tris (pH 8.4); 50 mM KCl; 2.5 mM MgCl₂; 16 pmole of each primer; 50 µM each ATP, CTP, GTP, and TTP; 1.5 U *Taq* polymerase; and 100 ng total DNA. Polymerase chain reaction amplification was performed on a Stratagene Robocycler (Stratagene Corporation). Amplification products were purified with spin columns (Qiagen Corporation, Valencia, CA, USA) and quantified via gel electrophoresis. Sequencing reactions were performed using ABI Cycle-Sequencing kits (Perkin-Elmer/ABI Corporation, Norwalk, CT, USA) using LGL 283 as the sequencing primer. Sequencing reaction products were analyzed on ABI Prism model 373 or 377 automated sequencing apparatus (Perkin Elmer/ABI). The DNA sequences were assembled and aligned using Sequencher® software (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences with ambiguous base calls were resequenced, using reamplified polymerase chain reaction products if necessary. Any sites with ambiguous calls that could not be resolved in this manner were excluded from the analysis. A total of 257 bases were used for this analysis. Sequences were compared to those reported in GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) to verify that amplification products were of rodent origin and not the result of contamination.

Genetic diversity. Genetic diversity within populations was calculated using nucleotide diversity indices and associated variances [20], with the Jukes–Cantor correction for multiple hits [21]. Statistical significance was calculated using *t* tests as described previously. Percentage diversity attributable to within- versus among-population variation was calculated using the software program AMOVA [22]. An additional measure of variability was calculated using this program as $SS_i / (n_i - 1)$, where SS_i and n_i are the within-population sum of squares and sample size, respectively, for population *i*. Bartlett's statistics (*B*) were calculated between all pairs of populations [23], and statistical significance (of among-population differences in variability) was assessed by random sampling from a pool of individuals from all populations, calculating *B* for 1,000 iterations. The significance level was taken to be the probability that the observed *B* was greater than expected from random sampling.

Genetic distance and gene flow. Comparisons among populations included genetic distance, population subdivision, and gene flow. Genetic distances were calculated as the average number of nucleotide substitutions per site among populations (with a Jukes–Cantor correction for multiple hits) and associated variances [24]. These distances were then used to construct population dendrograms by the neighbor-joining method [25]. Population subdivision was computed using Φ_{st} (an analog of *F_{st}*) statistics computed using the AMOVA analysis software [22]. Calculations included both total and among-population Φ_{st} . Statistical significance was calculated by random sampling from a pool of individuals from all populations for 1,000 iterations as described previously for Bartlett's statistics.

Patterns of migration were also ascertained by constructing a haplotype phylogeny, using the neighbor-joining method

with Tamura–Nei [26] distances. The phylogenetic tree was rooted using *D. microps* (collected from site R1) as the out-group (GenBank accession AF136529). The minimum number of migration events among populations needed to explain the topology of the tree was determined according to Slatkin and Maddison [27]. This method initially entails examination of the haplotypes at the terminal nodes of the tree. If two or more populations share this haplotype, then it is assumed that at least one migration event has occurred. Then one moves from the branch tips recursively toward the root. Each node of the tree is then assigned a state set derived from the set of sampling locations, each character state being the designation for one or more of the sampling sites. For example, possible states for the present study include R1, R2, T1, T4, R1/T1, R1/T4, T1/T4, R1/T1/T4, and so on. The state set of each node is determined from the state sets of the nodes that join to form it. If a node is formed by the union of an R1 node and a T1 node, then its state set is R1/T1, indicating that a minimum of one migration event between R1 and T1 is needed in order to account for this topology. When a node is formed by the union of a T1 and an R1/T1 node, the state of this new node is taken to be T1 (this is determined by simple majority rule), and it is inferred that the R1/T1 node represents at least one migration event from T1 to R1 (the new node represents the ancestral state of the T1 and R1/T1 nodes). Conversely, if the union of an R1 and an R1/T1 node formed a new node, then the new node is assigned a state of R1, and the migration event was assumed to have been from R1 to T1.

Terminal branch haplotype analysis. The terminal branch haplotype (TBH) procedure was performed according to [9]. Haplotypes were designated as TBHs only if they were located on the terminal branches of the phylogenetic tree and differed from the ancestral haplotype by one nucleotide (ancestral and derived states are inferred from the topology of the tree) [9]. For each population, a TBH index was then calculated as the number of TBHs in each sample divided by sample size [9]. Differences among populations were determined by calculating a standard normal statistic with comparison to the standard normal distribution [28].

Clastogenesis versus haplotype

Haplotype analysis of the different populations revealed that some haplotypes were unique to each population, while others were shared among populations (see the Results section). It was surmised that individuals with the unique haplotypes were probably long-term residents of that population, while at least some of the individuals with shared haplotypes may be immigrants from other populations (see the Discussion section). Because migration could affect the relative amounts of clastogenic damage, both contaminated populations were pooled, as were both reference populations. Micronuclei counts and DNA content CVs were then compared between individuals with shared versus unique haplotypes and tested with the Wilcoxon rank sum test. For this analysis, if significant differences were found, then comparisons among the four populations were performed first with all individuals and then only with individuals possessing haplotypes unique to each population using the Kruskal–Wallis test with multiple comparisons.

RESULTS

Radiation dosimetry

Radiation exposure levels at the T1 and T4 sites were 92 ± 43.3 and 346 ± 353 (mean \pm SD) $\mu\text{R}/\text{h}$, respectively. Be-

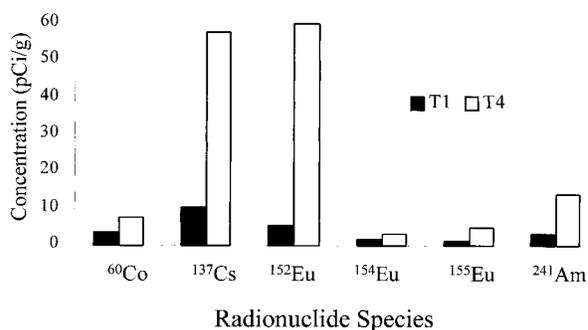


Fig. 2. Concentrations of selected radionuclides from two ground-zero atomic blast sites (T1 and T4) at the Nevada Test Site. Bars represent median values of the concentrations reported in [31,32] and are reported here only for samples from the top 2.5 cm of soil.

cause of the physical properties of ionizing radiation particles, γ radiation probably comprised the bulk of the external exposure. Although body burden analysis was not performed, there is the potential for accumulation of radionuclide ingestion and bioaccumulation of internal α , β , and γ radiation emitters [29].

Sauls et al. [30] employed dosimeter collars to estimate external radiation doses in *D. merriami* living near ground-zero T2 (a nearby detonation site). They found that animals living within 150 m of ground zero were receiving γ ray doses in excess of 1 R per month, on average, with additional β doses. Given similarities in the physical characteristics of the substrates at ground-zero T1, T2, and T4, it is likely that kangaroo rats at T1 and T4 experienced similar dosage rates. In addition, Sauls et al. [30] found that animals 1.5 km from T2 received doses of 17 to 38 mR per month, so it is likely that the reference populations receive doses considerably lower than the ground-zero populations. Other surveys [31,32] have also determined radionuclide concentrations in T1 and T4 soil at levels much higher than background (Fig. 2).

Clastogenic damage

Micronucleus assay. Sexes were pooled for all genotoxicity analyses because no significant differences were observed between males and females ($p > 0.05$ for all comparisons, Wilcoxon rank sum test). When all individuals were included in the analyses, no statistically significant differences were observed among populations (Fig. 3A; $p > 0.05$, Wilcoxon rank sum test). However, when the analysis was limited to individuals with haplotypes unique to each population, the average number of micronuclei was significantly greater in T4 than in R1 and R2 (Fig. 3A; $p < 0.05$). No other statistically significant differences were detected.

The individuals with haplotypes that were unique to at least one contaminated site had a higher average number of micronuclei than did the individuals with haplotypes shared with at least one reference population (Fig. 3C; $p < 0.05$). No such differences were found between individuals with haplotypes unique to the reference sites versus shared with contaminated sites ($p > 0.05$).

Flow cytometry. There were no statistically significant differences in CV among any of the sites (Fig. 3B; $p > 0.05$, Kruskal–Wallis test). However, when the analysis was limited to individuals with haplotypes unique to each population, the difference between the T4 and R1 populations was significant (Fig. 3B; $p < 0.05$, Kruskal–Wallis test). Moreover, within the

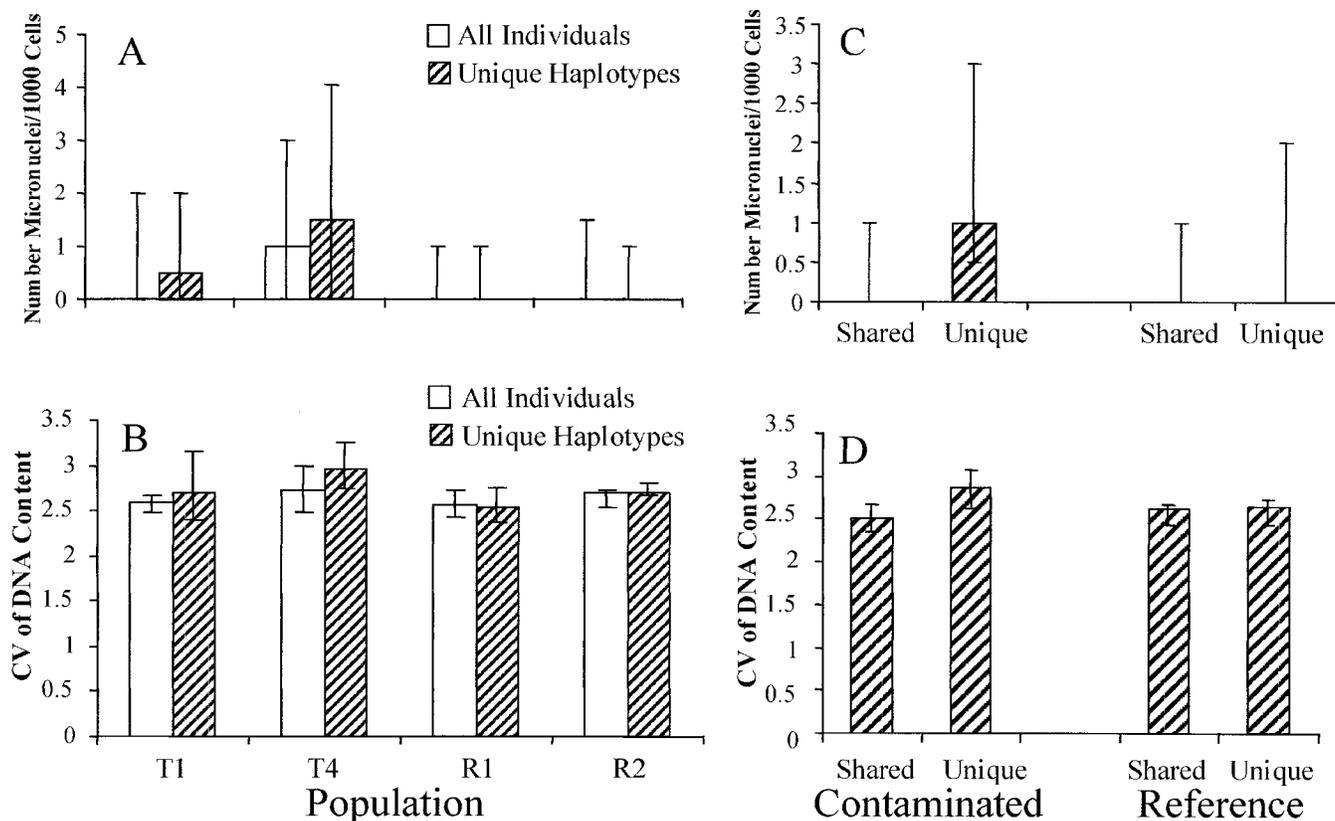


Fig. 3. Genotoxic effects of radiation exposure (micronuclei and half-peak coefficient of variation [CV] of DNA content) from four populations of kangaroo rats. Populations R1 and R2 are uncontaminated reference sites, and populations T1 and T4 are ground-zero atomic blast sites at the Nevada Test Site. (A) and (B) show data for all populations. (C) and (D) show comparisons of pooled reference and contaminated sites, with animals possessing mitochondrial DNA (mtDNA) haplotypes found in both reference and contaminated populations (shared) compared to animals with haplotypes found only at the reference or contaminated sites (unique). Significant and nonsignificant comparisons are explained in the Results section. Bars and error bars are medians and quartiles, respectively. The lack of a bar for any group indicates that the median = 0.

contaminated sites, individuals with haplotypes unique to at least one contaminated population had higher CVs than did individuals with haplotypes shared with at least one reference site (Fig. 3D; $p < 0.05$, Wilcoxon rank sum test). In the reference populations, no differences were found between individuals with haplotypes unique to the reference sites versus shared with contaminated sites (Fig. 3D; $p > 0.05$).

RAPD analysis

Twenty-nine bands ranging from 300 to 1,500 bp were identified, 12 of which were polymorphic. The average number of bands per individual for populations R1, R2, T1, and T4 was,

respectively, 25.12, 24.85, 24.73, and 25.42. There were no significant differences between any of the sites in genetic diversity as measured by band sharing indices or gene diversity. Genetic distances based on band sharing between all pairwise comparisons of populations indicated that genetic distances were similar among all pairs of populations (Table 1). No significant differences were observed between contaminated and reference populations for band frequency differences (Table 2).

Table 1. Pairwise genetic distances^a (upper right) and Fst statistics^b (lower left) among four populations of *Dipodomys merriami* based on the randomly amplified polymorphic DNA (RAPD) technique. Populations R1 and R2 are uncontaminated reference sites, and populations T1 and T4 are ground-zero atomic blast sites at the Nevada Test Site, Nevada, USA

	R1	R2	T1	T4
R1	—	0.058	0.057	0.057
R2	0.012	—	0.052	0.055
T1	0.018	0.031	—	0.056
T4	0.006	0.019	0.025	—

^a Genetic distances were calculated as $1 - S_b$, where S_b is the average similarity index between individuals in the two populations [21].
^b Calculated according to Lynch and Milligan [19].

Table 2. Frequencies of the seven most highly polymorphic randomly amplified polymorphic DNA (RAPD) bands^a in four populations of *Dipodomys merriami*. Populations R1 and R2 are uncontaminated reference sites, and populations T1 and T4 are ground-zero atomic blast sites at the Nevada Test Site, Nevada, USA

RAPD band	Population			
	R1	R2	T1	T4
OPD2 _{1,420}	0.038	0.0	0.067	0.032
OPD2 _{1,390}	0.423	0.153	0.267	0.378
OPD2 _{1,010}	0.846	0.769	0.867	0.968
OPD7 _{1,370}	0.269	0.307	0.200	0.354
OPD8 ₆₈₀	0.769	0.692	0.733	0.580
UBC2 _{1,430}	0.538	0.461	0.533	0.645
UBC12 _{1,240}	0.423	0.461	0.573	0.451

^a Band names are the names of the primer (designated by the manufacturer) followed by the molecular length (kb) in subscript. Nucleotide sequence for each primer is given in the text.

Table 3. Designations for 41 mitochondrial DNA (mtDNA) haplotypes based on their sequence variation at 27 variable positions. Periods denote identity with haplotype H1. The identification of all individuals possessing each haplotype is given, as is the locality from which each was taken, in Figure 6

Haplotype	Sequence of polymorphic sites
	111111111122222222222222
	113111145568901112233334444
	122015620268210361924572345
H1	GGTGATATGGGTGTTGGTGGAGGTAGG
H2	..C.....A.....C.....G..
H3A.....
H4C.....
H5G.A.....A.....G..
H6A.....
H7TA.....
H8G.....C.....
H9A.....C.A.....
H10C.....G..
H11A.....C.....G..
H12A.....C..G..CG..
H13	..C.....A.....G..CA..G..G..
H14C.....A.....
H15A.....G.A.....
H16A.....G.....
H17C.....G.....
H18	..A.A.....A.....G.....
H19C.....T..
H20G.....
H21G.....
H22G.....
H23A.....C.....G..
H24A.....C.....C..G..G..
H25C.....C.....G..
H26	..C.....A.....A..C.....G..
H27	..C.....A.....A..C.....G..
H28A.....AAT..T..
H29AA.....C.....G..
H30	..C.....A.....A..C..G..G..
H31AA.....A..C..G..G..
H32A.....A..C..G..G..
H33A..AA.....C.....G..
H34	..C.....A.....G..C..G..G..
H35	..C.....G.A.....A.....G..G..
H36	..C.....G.A.....A.....G..G..
H37	A.....AG.A.....AC.....G..
H38	..C.....GAA.CA..AAC..G..G.A
H39GAA.CA..AC..G..G..
H40	..A.A.....AAA.CAA..A.A.....G..
H41	..A.A.....AAAA.AG..A.A.....G..

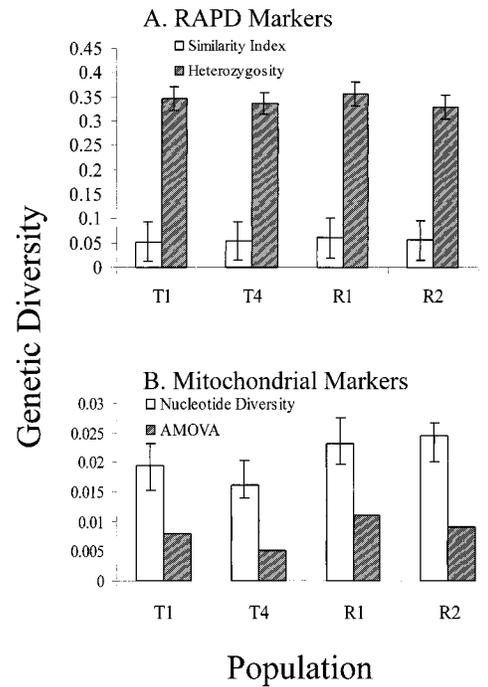


Fig. 4. Genetic diversity of four kangaroo rat populations measured using randomly amplified polymorphic DNA (RAPD) (A) or mitochondrial DNA markers (B). Diversity, as determined using similarity indices, was measured as $1 - S_w$, where S_w is the average similarity index between all possible pairs of individuals within each population. Nucleotide diversity is an index (and associated variance) calculated according to methods in [20]. Analysis of molecular variance software (AMOVA) was used to calculate among- and within-population diversity [22] and is determined as the sum of squares within each population divided by $(n - 1)$, where n is the sample size of each population. Populations R1 and R2 are uncontaminated reference sites, and populations T1 and T4 are ground-zero atomic blast sites at the Nevada Test Site.

Mitochondrial DNA analysis

A 257-bp section of the control region was used for the analysis (GenBank accession no. AF136528). This section was complementary to the *Mus musculus domesticus* control region, positions 371 to 641 (GenBank accession no. U47464). A total of 41 haplotypes were observed from all populations. The sequence of the variable positions and the individuals possessing each haplotype are reported in Table 3. The se-

Table 4. Mitochondrial DNA haplotypes present in four populations of *Dipodomys merriami*, the total number of haplotypes unique to each population, and the total number of individuals possessing unique haplotypes. Populations R1 and R2 are uncontaminated reference sites, and populations T1 and T4 are ground-zero atomic blast sites at the Nevada Test Site, Nevada, USA

Population	n	Haplotypes present (total number)	Unique haplotypes	Individuals with unique haplotypes
R1	24	H1, H2, H3, H5, H6, H7, H8, H10, H11, H12, H13, H14, H17, H27, H28, H29, H32, H38, H39 (19)	7	7
R2	9	H5, H7, H24, H30, H33, H34, H35, H36, H41 (9)	7	7
T1	11	H1, H6, H8, H13, H18, H25, H26, H40 (8)	3	3
T4	24	H1, H2, H3, H4, H9, H10, H11, H12, H14, H15, H16, H19, H20, H21, H22, H23, H31, H37 (18)	11	14

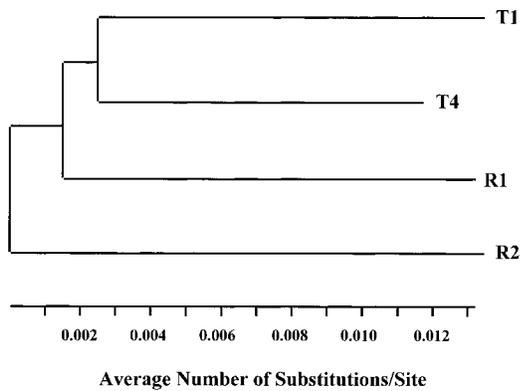


Fig. 5. Neighbor-joining tree showing the relationships among four populations of kangaroo rats (*Dipodomys merriami*). The tree was calculated from mitochondrial DNA (mtDNA) control region sequences using a distance matrix of average number of nucleotide substitutions per site among populations. Populations R1 and R2 are uncontaminated reference sites, and populations T1 and T4 are ground-zero atomic blast sites at the Nevada Test Site.

quence of haplotype H1 is 57.2% A-T. Twenty-seven polymorphic sites were detected, 19 of which were parsimony informative. There were 19 transitions (including 16 G ↔ A and 3 C ↔ T) and one transversion (G ↔ T at site 244). There were also seven sites that contained three nucleotides: site 32 (C, G, T); sites 142, 150, 201, and 210 (A, G, T); site 115 (T, C, A); and site 216 (A, C, G). The total number of haplotypes in each population, number of unique haplotypes per population, and number of individuals with haplotypes unique to each population are summarized in Table 4.

Genetic diversity. There were no statistically significant differences among populations in terms of the amount of nucleotide diversity (Fig. 4). However, the amount of within-population diversity, as calculated using AMOVA, was lower in population T4 than in R1, but no other significant differences were observed (Fig. 4). The AMOVA also indicated that most of the diversity occurred within groups (96%) rather than among groups (4%). The overall Φ_{st} estimate was 0.039, which was significantly different from one computed from a sample selected randomly from all individuals in the four populations ($p = 0.011$).

Genetic distance and gene flow. Neighbor-joining analysis of the genetic distance data revealed that the T1 and T4 populations were more closely related to each other than to the reference sites and that population R1 was more closely related to the contaminated sites than was R2 (Fig. 5). Neighbor-joining analysis of the haplotype sequences indicated that there was no clear geographic pattern in haplotype distribution (Fig. 6). Migration events necessary to explain the topology of the tree in Figure 6 are indicated by black diamonds. When two populations shared a haplotype, the probable origin of that haplotype was estimated from the phylogenetic tree of the haplotypes. Probable direction of migration events was discerned in the same way. Using this analysis, we found that 27 migration events are needed to explain the tree (Table 5), 23 of which the direction of migration can be determined. Of these, 13 migration events involved movement of animals from the reference areas into the contaminated areas, and six involved migration from the contaminated areas into the reference areas. The remaining migration events were exchanges within contaminated or reference areas. The greatest number

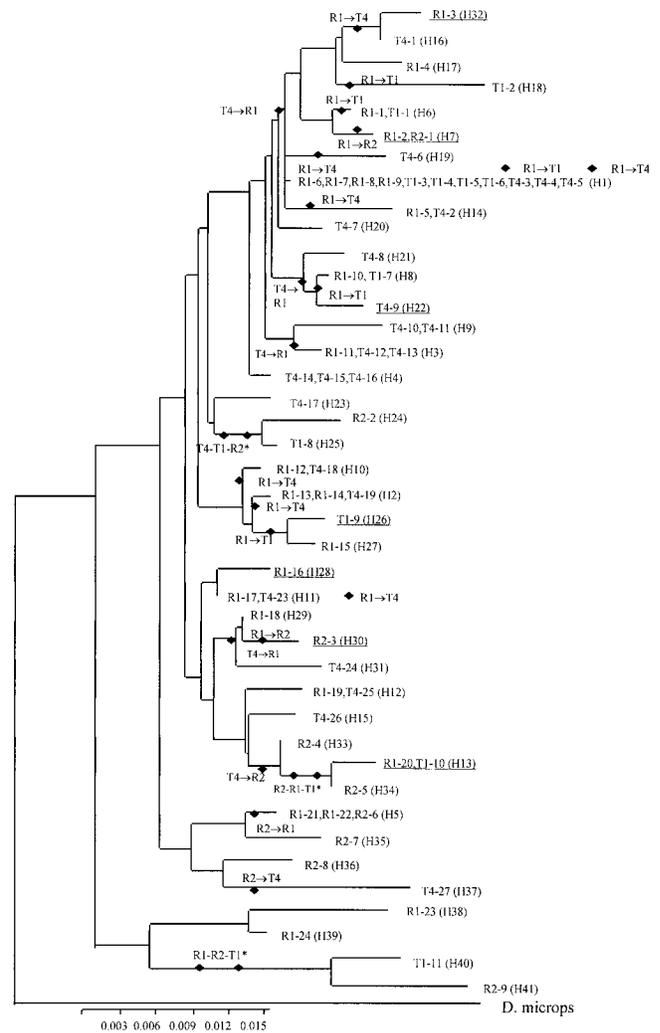


Fig. 6. Neighbor-joining tree for 41 mtDNA haplotypes based on control region sequences for kangaroo rats (*Dipodomys merriami*). The tree is based on Tamura-Nei distances. Individuals with each haplotype are listed (e.g., R1-12 refers to individual 12 from population R1), followed by the haplotype number in parentheses (Table 3). Diamonds represent ostensible migration events needed to explain the topology of the tree as explained in the Results section. Terminal branch haplotypes are underlined. Populations R1 and R2 are uncontaminated reference sites, and populations T1 and T4 are ground-zero atomic blast sites at the Nevada Test Site.

of migration events involved animals migrating from R1 → T4 ($n = 7$) and R1 → T1 ($n = 5$).

We calculated Φ_{st} estimates for all pairwise population comparisons. The only estimates that were significantly different from one computed from a sample selected randomly from all individuals in the four populations were between R2 and T1 ($\Phi_{st} = 0.116$) and between R2 and T4 ($\Phi_{st} = 0.155$).

Terminal branch haplotype analysis. There were no statistically significant differences between populations for the TBF or TBH index, although the values for the reference sites were higher than the ground-zero sites, particularly T4 (Table 6).

DISCUSSION

The primary objective of this study was to assess the environmental impact of radionuclide contamination on Merriam's kangaroo rats by integrating analyses of genetic response at somatic and population genetic levels. Previous studies of vertebrate populations exposed to radiation in contaminated

Table 5. Estimated number of migration events^a among four populations of kangaroo rats (*Dipodomys merriami*). Migration is into the populations listed on the top row and out of the populations listed on the left column. Populations R1 and R2 are uncontaminated reference sites, and populations T1 and T4 are ground-zero atomic blast sites at the Nevada Test Site, Nevada, USA

	R1	R2	T1	T4
R1	—	2	5	7
R2	1	—	—	1
T1	—	1	—	—
T4	4	1	1	—

^a As per [27]; see Figure 6.

environments have demonstrated significant somatic effects [3]. However, initial analyses of somatic data for the kangaroo rats did not demonstrate significant effects for either flow cytometric or micronucleus assays. One possible explanation for the absence of a genotoxic response at the contaminated sites is the influence of immigration from noncontaminated populations nearby. Recent immigrants would not have the exposure history of resident individuals and thus are less likely to show chronic effects of radiation (chromosomal damage). External dose rates of γ radiation at the T sites are relatively low, such that this venue of exposure may not contribute significantly to chromosomal damage. Nonetheless, radionuclide body burdens of kangaroos rats inhabiting the T sites are influenced by uptake and accumulation through their diet [29]. Thus, internal dose rates should correspond to residence time at these sites, with a greater potential for detectable chromosomal damage in long-term residents than in recent immigrants. (The phrase “long-term resident” is relative to the life span of *D. merriami*, about three to four years [12].) Immigration from noncontaminated sites could increase intrapopulation variation and decrease interpopulation variation, thereby obscuring a population-level response to exposure at the T sites. By examining population genetic structure among contaminated and reference sites, we were able to address the potentially confounding influence of immigration on genotoxicity at the T sites.

We assumed that animals collected at the contaminated sites might include both residents and recent immigrants. We hypothesized that animals with mtDNA haplotypes found only at the contaminated sites were most likely to be residents, while animals with haplotypes found also at the reference sites might be immigrants. The theoretical basis for this hypothesis is well established. For example, Slatkin [33] devised a method to estimate gene flow among populations using the frequencies of private (unique) alleles as an indicator of gene exchange. Private alleles are those that are not shared among populations and whose numbers and frequencies are determined by the degree to which populations are isolated. That is, gene flow resulting from migration reduces the proportions of private alleles in populations. In our study, we have no direct evidence that the animals with shared haplotypes represent migrants, and to obtain such data would require an exhaustive demographic survey of the populations. Nonetheless, the fact that the shared haplotype individuals differed significantly for both indicators of genetic damage compared to the unique haplotype (private allele) individuals is strong evidence that the former group is comprised at least partially of immigrants. The probability of the results of both tests being due to sampling error is remote.

The mtDNA data also help to discern the pattern of mi-

Table 6. Number of terminal branch haplotypes (TBH) per population, frequency of terminal branches (TBF), and terminal branch haplotype index for four populations of kangaroo rats (*Dipodomys merriami*). Populations R1 and R2 are uncontaminated reference sites, and populations T1 and T4 are ground-zero atomic blast sites at the Nevada Test Site, Nevada, USA

Population	No. TBH	No. individuals with TBH	Ratio of TBH/H ^a	TBI (TBH/n) ^b
R1	4	4	0.200	0.182
R2	2	2	0.250	0.222
T1	2	2	0.250	0.133
T4	1	1	0.059	0.042

^a Number of terminal branch haplotypes \div total number of haplotypes.

^b Number of terminal branch haplotypes \div total number of individuals.

gration among sites. A high level of gene flow was detected, especially between R1 and the ground-zero sites. Values of *F*_{st} for both mtDNA and RAPD analyses were low, suggesting little if any population subdivision among sites R1, T1, and T4. Corroborative findings were observed in the migration analysis, where a neighbor-joining tree of haplotypes was used to calculate the minimum number of migration events among populations. The tree's topology implies a number of migration events between R1 and the ground-zero sites and suggests that gene flow is predominantly from R1 into the T sites (Fig. 6). Despite geographic proximity, fewer migration events can be inferred between T1 and T4, whose haplotypes shared with R1 lie within branch networks dominated by R1 haplotypes (Fig. 6).

Another mechanism that may account for the low *F*_{st} values observed among populations involves founder events following extirpation at the ground-zero sites. The T1 and T4 sites were recolonized after nuclear weapons tests, and our genetic data suggest that the founders were related to the R1 and, possibly, R2 populations (either through common ancestral haplotypes or through recent gene flow). Again, topological patterns in the neighbor-joining tree, where assemblages are deeply rooted by haplotypes found only in R1 and R2, support this contention. Other studies reporting little or no genetic differentiation among *Dipodomys* populations offer local extinction and recolonization as a likely explanation [34]. These processes can reduce or eliminate genetic differences among populations, especially if the number of founders is large relative to the migration rate [35].

Are the low *F*_{st} values and shared haplotypes in *D. merriami* due primarily to ground-zero extirpation and historical recolonization? Patterns in mtDNA variation indicate that shared haplotypes are not due solely to extinction/recolonization events. The relative age of alleles (haplotypes) is correlated with their position on a phylogenetic tree [36], such that ancestral haplotypes are deeply rooted topologically and those more recently derived reside nearer terminal branches. If haplotypes shared between ground-zero and reference sites reflect coancestry through founder events, then they should be deeply rooted in the tree. However, many of the shared haplotypes are at the branch tips, whereas some of the unique haplotypes are deeply rooted (Fig. 6). Thus, it appears that the low *F*_{st} values and shared haplotypes are not due to extinction/recolonization alone but probably involve recent dispersal among populations. We conclude that migration occurs among the sites and that the majority of recent gene flow has been from R1 to T1 and T4. Given the evidence for genetic ex-

change, we stress the need to examine the genotoxicity data in light of population genetic data for possible impacts of immigration on genetic responses to exposure. Had this study been based on the genotoxicity data alone, signals for chromosomal damage would have gone undetected.

Another aspect of migration that could influence detection of genotoxic effects involves differential dispersal between males and females. Differential dispersal can generate different patterns of genetic structure for analyses of mitochondrial (maternal) versus nuclear (biparental) markers, especially if dispersal is male biased [37]. The kangaroo rat populations showed no subdivision for RAPD markers, but a significant *F*_{st} was observed for mtDNA haplotypes between the contaminated and R2 sites. *Dipodomys merriami* has male-biased dispersal [12] and thus exhibits a behavioral profile that is consistent with our population genetic data.

A potential application of population genetics in ecotoxicology involves the investigation of selection and adaptation to contaminant stress. Previous studies employing RAPD markers have provided evidence for pollutant-influenced selection in populations of mosquitofish exposed to radionuclides [11,15]. We used the same RAPD primers to examine the ground-zero populations of *D. merriami*, but no consistent differences were detected between the contaminated versus reference populations. It is possible that the RAPD primers amplified different loci in the kangaroo rats than in the mosquitofish. Alternatively, any effects of pollutant-induced selection may have been obscured by gene flow through immigration.

A final toxicological application of population genetics involves probing for pollutant-induced mutations. We found no evidence of elevated mutation rates in the contaminated populations. Other studies have examined control region sequences to assess mutation rates of organisms exposed to mutagenic PAHs, but results were equivocal [9]. Perhaps mutation rates in these exposed populations are not significantly higher than background or migration obscures mutagenic effects at this scale of population genetic comparison. Alternatively, processes other than mutation could influence the frequency of tip haplotypes in each population, such as population expansion or decline [38]. In any case, studies conducted to date, including this one, have not shown the terminal branch haplotype technique to be effective in detecting increased mutation rates in the mitochondrial control genome. The fact that present technology samples only a tiny fraction of the genome, however, necessitates caution in concluding that mutations are not induced by contaminant exposure [6].

The results of our investigation illustrate the varied and important contributions of population genetic analysis to ecotoxicology studies and environmental risk assessments. These techniques can be used not only to examine the effects of pollutants on genetic diversity and genotypic distributions [2,39] but also to help ascertain how dispersal and migration may impact the biological effects of contamination. By identifying and compensating for these phenomena, researchers can enhance the resolution of biomarker assays using natural populations. Indeed, our initial genotoxicity analyses suggested that there was no significant effect of radiation exposure at the ground-zero sites, whereas subsequent integration of the population genetic data suggests that a portion of the ground-zero populations are experiencing significant genotoxic effects and that the ground-zero sites may act as migration and gene

flow sinks. The approaches described herein should find wide application in future studies of ecotoxicology.

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