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Estimates of mtDNA Mutation Frequencies in Wild White-footed Mice Based on Terminal Branch Haplotype Analysis

Mathew Camaioni[1], Brian Hjelle[2] and *Sabine S. Loew[1]
[1]Department of Biological Sciences
Behavior, Ecology, Evolution and Systematics Section
Illinois State University, Normal, Illinois, USA
[2]Center for Emerging Infectious Diseases
Infectious Diseases and Inflammation Program, Department of Pathology
University of New Mexico School of Medicine, Albuquerque, New Mexico, USA

*correspondent: ssloew@ilstu.edu

ABSTRACT

We applied terminal branch haplotype (TBH) analysis of mitochondrial DNA in wild white-footed mice (*Peromyscus leucopus*) to estimate mutation rates as a measure of genotoxic impact of environmental pollutants. Mice were sampled from two heavy metal contaminated areas at Superfund Sites and two reference sites in northern and southern Illinois. The geographic distribution of mitochondrial DNA haplotypes showed a clear north-south split, however, we found no statistically significant difference in mutation rates as estimated by TBH indices for mice derived from contaminated versus reference sites. These results could be due to mutagen exposure below the critical threshold, or may be indicative of low sensitivity of mtDNA as a molecular marker for genotoxicity.

Key Words: Germline mutation, Hantavirus, Heavy metal, Superfund Sites, Mitochondrial DNA, *Peromyscus leucopus*, Terminal branch haplotype

INTRODUCTION

As abundance of contaminants in the environment has increased, so has exposure of wildlife to those contaminants (Nriagu and Pacyna 1988). Accumulation of heavy metals, organohalogens and other toxicans has been documented in organisms at skeet ranges (Ma 1989; Stansley and Roscoe 1996), military installations (Dickerson et al. 1994; Simini et al. 1995), mining and industrial sites (Bargagli et al. 1997; Laurinolli and Bendell-Young 1996; Lepage and Parker 1988; Pascoe et al. 1994; Tull-Singleton et al. 1994) and among other habitats (Shugart and Theodorakis 1994). Frequently pollutants are present as complex mixtures that present organisms with multiple stressors (Clark et al. 1992; Facemire et al. 1995; Newman and Unger 2003; Shore and Rattner 2001). Few wildlife studies have focused on chronic genetic effects of environmental contaminants,

even though a number of these pollutants are known or suspected genotoxins that cause physical damage (e.g., chromosome breakage, DNA adducts) or mutagenic damage (e.g., nucleotide substitutions, insertion/deletion of nucleotides) to DNA molecules (Ariza and Williams 1996; Butterworth et al. 1995; Hedenskog et al. 1997; Moore et al. 1997).

Genotoxicity studies frequently fail to address two areas: effects of a biologically relevant exposure level and long-term heritable effects. Most studies are based on mutagenic assays performed under controlled lab conditions with cell cultures or lab-bred animals to which predetermined amounts of a suspected chemical are administered (Ariza and Williams 1996; Ledwith et al. 1995; Ogheri et al. 1995). Although some studies make attempts to simulate naturally occurring levels of pollutants present in the environment (Ariza and Williams 1996; Hedenskog et al. 1997), the concentrations of mutagens frequently far exceed those of contaminants present in the environment in order to enhance the treatment effect (Butterworth et al. 1995; Ogheri et al. 1995). Such approaches are essential to determine genotoxic chemicals and to provide guidelines for public health and industrial exposure limits, but they often are limited in assessing risk to exposed wildlife or human populations (Dickerson et al. 1994). For example, natural populations may be exposed to complex mixtures of contaminants, or multiple routes of exposure (e.g., ingestion of contaminated soil due to grooming and foraging) and may vary greatly among species (Beyer et al. 1994). In order to evaluate biologically relevant exposure levels and typical routes of exposure, organisms must be sampled in their natural environment.

Previous ecotoxicological studies have tended to use large-scale measures of DNA alterations (Ieradi et al. 1998; Shugart and Theodorakis 1994; Thies et al. 1996). While useful in monitoring contaminant exposure and genotoxic effects, these measures are not sensitive enough to detect alterations at the molecular level and do not adequately address any increase in the rate of molecular mutations due to contamination. As new molecular techniques became routinely available (e.g., polymerase chain reaction, DNA sequencing), they have been used to examine consequences of genotoxic substances on exposed wildlife (Baker et al. 1996, but see Baker et al. 1997; Chen and Hebert 1999a,b; Ellegren et al. 1997; Somers et al. 2002; Yauk and Quinn 1996; Yauk et al. 2000).

Mitochondrial DNA (mtDNA) sequencing offers another tool to assess genetic damage and its consequences due to environmental contaminants. Several studies have indicated that mtDNA is sensitive to environmental contaminants. Mitochondrial DNA shows much higher levels of DNA adduct formation (Allen and Coombs 1980; Backer and Weinstein 1980) and oxidative DNA damage than nuclear DNA (Richter et al. 1988; Yakes and Van Houten 1997). Mitochondrial DNA, especially the noncoding D-loop (i.e., control region) is ideally suited for phylogenetic reconstruction and estimates of molecular change (Brown 1985; Parsons et al. 1997). The D-loop region is highly variable and is considered the most rapidly evolving region of the mtDNA genome (Saccone et al. 1987). D-loop regions that neither code for proteins nor have a regulatory function are expected to be selectively neutral, ensuring that mutations will not be eliminated from the gene pool, hence they can be detected.

Chen and Hebert (1999a,b) used the variable, selectively neutral D-loop region of mtDNA and developed a novel approach that allowed discrimination between recently

derived mutations and ancestral polymorphism. Because intergenerational mtDNA differences usually arise as single nucleotide changes (Parsons et al. 1997) and newer genealogical derivatives may be separated by only a single or a few generations, Chen and Hebert (1999b) defined terminal branch haplotypes (TBHs) as recently arisen haplotypes (i.e., terminal branches of a phylogeny) that differ from their ancestral haplotypes by a single nucleotide change. They were able to identify TBHs by combining DNA sequencing with phylogenetic analysis. They applied TBH analysis to examine effects of environmental pollutants in brown bullhead (*Ameiurus nebulosus*), which inhabited the same area as herring gulls (*Larus argentatus*) that had increased rates of nuclear minisatellite mutations (Yauk and Quinn 1996). Chen and Hebert (1999b) demonstrated the efficacy of TBH analysis in detecting novel mutations of recent origin. In our study, we modified their TBH approach to examine the frequency of novel mutations in white-footed mice (*Peromyscus leucopus*) that inhabit sites contaminated by heavy metals and corresponding reference sites in Illinois.

MATERIALS AND METHODS

Study species

The white-footed mouse (*Peromyscus leucopus*) is a medium-sized species of *Peromyscus* that is found from southern Canada to southern Mexico east of the Rocky Mountains (Baker 1968). While present in almost every habitat of Illinois, white-footed mice prefer wooded or brushy areas (Hoffmeister 1989). Their population density in Illinois varies depending on location and season (Hoffmeister 1989), but it is sufficiently large to make the species ideal for environmental and genotoxicological assessment. *Peromyscus*, including white-footed mice, have been used previously to study the uptake of environmental contaminants (Clark et al. 1992; Laurinolli and Bendell-Young 1996; Pascoe et al. 1994; Stansley and Roscoe 1996; Tull-Singleton et al. 1994) as well as the potential genetic consequences of such contaminants (McBee and Bickham 1990). Since white-footed mice have been found to disperse up to 300 m (Keane 1990), we made sure that paired contaminated and reference sites were separated by at least 900 m to minimize the possibility of gene flow.

Wild mice were trapped from July to October, 1999 and May to October, 2000 using baited Sherman live-traps. To account for the risk of hantavirus from potentially infected wild mice, we followed published protocols and recommendations for trapping and handling (Mills et al. 1995a,b) as well as quarantine of the mice (Bharadwaj et al. 2000; Botten et al. 2000; Camaioni et al. 2001). In this study we used 46 mice, all of which had tested negative for Sin Nombre virus. Animals were sacrificed under deep anesthesia by cervical dislocation; their carcasses or skeletons were kept as voucher specimens for future reference. All animals were treated in accordance with locally accepted IACUC protocols (ISU IACUC protocol #1-98-12).

Sample sites

White-footed mice were trapped at two contaminated and two reference sites in Illinois (Figure 1 and Table 5). The first contaminated site (i.e., site DePue) was located in northern Illinois on municipal property of the Village of DePue, directly northeast of the DePue/New Jersey Zinc/Mobil Chemical Corporation (NJZMC) site in Bureau County, Illinois. The latter is the location of a former zinc smelter that was declared a Superfund

site and placed on the USEPA National Priorities list by 1999 (US EPA FRL-6338-5 1999). This New Jersey Zinc/Mobil Chemical (NJZMC) site consists of approximately 324 hectares of land in the northeastern part of the Village of DePue. Ecotoxicological assessments by the EPA showed significantly elevated levels of heavy metals (e.g. cadmium, copper, lead, mercury and zinc) in soil and sediment samples taken from DePue Village and the NJZMC industrial site, which ultimately resulted in its designation as Superfund site (IL EPA 1992). The second contaminated site (i.e., site P1) was located in the eastern portion of the Crab Orchard National Wildlife Refuge (CONWR) in Williamson County in southern Illinois. The Crab Orchard National Wildlife Refuge (CONWR) is a 43,000-acre area managed by the U.S. Fish and Wildlife Service. The eastern part of the refuge was occupied for over 60 years by a variety of manufacturing facilities, which resulted in contamination with PCBs, lead and other heavy metals and contaminants related to munitions and explosives manufacturing (Environmental Science and Engineering, Inc. 1994; McKee 1992; US EPA FRL-3187-6 1987). Therefore the eastern half of CONWR was listed as a Superfund site on the USEPA National Priorities list in 1987 (US EPA FRL-3187-6 1987). Several studies documented contaminant concentrations and uptake by wildlife at CONWR (Hite and King 1977; McKee 1992; Woolf et al. 1983). One such study specifically noted the bioaccumulation of lead and PCBs in *P. leucopus* (McKee 1992). Assuming an average generation time of 45 days for white-footed mice and a five months yearly breeding season (King 1968), the long-term presence of potential mutagens at DePue (approximately 100 years) and CONWR (approximately 60 years) could have resulted in exposure of 300 and 200 generations of white-footed mice, respectively.

Reference sites in the north (i.e., GL), and south at CONWR (i.e., CS) were well removed from sources of heavy metal contamination. The Goose Lake site (GL) is located on Goose Lake Prairie State Natural Area (GLPNA), 10 km east of Morris in Grundy County, IL and within 100 km of the contaminated site at DePue. Goose Lake Prairie State Natural Area is the largest remnant prairie remaining in Illinois. The southern reference site CS was located in the western part of the CONWR refuge, about 8 km from the contaminated areas of the eastern half of CONWR. This part of the refuge historically has been free of industrial development and has been managed solely as a wildlife and recreation area since the creation of the refuge.

Soil and tissue analysis for heavy metals

In order to assess the actual levels of heavy metal contaminants, ten soil samples were collected along parallel transects to achieve optimal sampling from each site at Goose Lake, DePue, and the southern control site CS. We used 8x15 cm hand-held stainless steel core sampler to collect soil in accordance with standard protocols (Illinois Department of Natural Resources 1999; Biser et al. 2004). We homogenized and dried all soil samples, which were subsequently analyzed for heavy metals by the staff of the Research and Laboratory Services Program (RLSP) at the Illinois Department of Natural Resources Waste Management Resource Center (IDNR-WMRC). They tested for cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), and zinc (Zn) based on Inductively Coupled Plasma Mass Spectroscopy (ICP-MS); mercury (Hg) concentrations were determined using Cold Vapor Atomic Fluorescence (CVAF) detection. Soil contaminant levels for the southern site P1 were derived from reports compiled by the U.S. Fish and Wildlife Service in compliance with EPA Superfund regulations (Vick 1999). To assess contami-

nant uptake in wild mice, we performed heavy metal analysis of tissue for individuals from our study sites at DePue, P1 and CS; sample sizes were limited to five individuals per site and no tissue analyses were conducted for Goose Lake due to technical and financial constraints. Liver of each animal was collected, weighed to ±0.001 g and stored in acid-washed polypropylene cryovials suitable for subsequent metals analysis as described above.

Our choice of metals to analyze at each contaminated site was informed by published information for contaminants at these sites (Environmental Science and Engineering Inc. 1994; US EPA FRL 3187-6 1987; US EPA FRL-6338-5 1999; Vick 1999). Metals tested at the reference sites were, at a minimum, those that had been documented for its matched contaminated sites (see also Biser et al. 2004). Values are reported as mg/kg wet weight. Soil and tissue samples were analyzed according to standard protocols by the Illinois Department of Natural Resources Waste Management and Research Center (EPA Method 3051 1986; EPA Method 6020 1992; IDNR-WMRC 1999). Concentrations for each metal were compared between the matched experimental and reference sites; except for tissue samples from DePue, which were compared to the southern reference site CS since tissue samples from Goose Lake mice were not available. Because the assumptions for t-tests and ANOVAs could not be met, we utilized nonparametric Mann-Whitney tests for pairwise comparisons and Kruskal-Wallis tests for comparisons between multiple sites in the south; our initial project design included two southern sites (P1 and P4) that were contaminated acc/to the literature, however, we do not present P4 results here as the Kruskal-Wallis test showed elevated contaminant levels for P1, but not P4 when compared to the southern control site CS (SAS Institute, Inc. 1996; Zar 1999).

mtDNA sequencing

Left kidneys were collected for DNA extraction from tissue. The minced tissue was placed in lysis buffer (100mM Tris, 100mM EDTA and 2% SDS) and quickly frozen either in liquid nitrogen or on dry ice until storage at -80ºC. DNA for each mouse was extracted from ~25 mg of kidney tissue using the DNeasy Tissue Kit (Qiagen Inc.) (see Table 5 for sample sizes). Two highly variable mtDNA fragments in the replication control (D-loop-containing) region were selected for sequencing (Morzunov et al. 1998). Fragment 1 was amplified with the primer pair MD1 (5'-GTCTAGCTGGACTTTTCAAT TCAAGC-3') and 12ST (5'-GCATTTTCAGT-GCTTTGCTTTATTG-3') while the primers CBT (5'CCGCCATC-AACACCCAAAGCTG-3') and MR1 (5'-CCCTGAAGTAAG AACCAGA-TGCCTG-3') were used for amplification of fragment 2 (Morzunov et al. 1998). Fragments 1 and 2 were 384 bp and 427 bp in length, respectively. Mitochondrial DNA fragments were amplified following Morzunov et al. (1998) and sequenced on an ABI 310 automated sequencer (PE Applied Biosystems) according to standard protocols (Camaioni 2001).

Terminal branch haplotype analysis

Sequence alignment of each mtDNA fragment within and between sites was performed using Sequencher v4.1 (GeneCodes Corporation). Individuals with identical nucleotide sequences were classified as a single haplotype. Common haplotypes and nucleotide differences among haplotypes were determined for each mtDNA fragment by pairwise comparison using MacClade (Madison and Maddison 2000). Correct assignment was confirmed with the program TCS (Clement et al. 2000). Recent mtDNA variants were

determined based on the terminal branch haplotype (TBH) analysis of Chen and Hebert (1999b) with a few modifications. They specify that a haplotype must meet two criteria to be classified as a TBH. First, the haplotype should have a single nucleotide difference (i.e., base pair) from another haplotype within the pool. Secondly, the haplotype had to have been derived from a recent mutational event as indicated by phylogenetic analysis. Single unit insertion and deletion rearrangements (e.g., one base pair deletion, insertion of a dinucleotide repeat in a microsatellite region) were also defined as TBHs if phylogenetic analysis determined that the rearrangement was recently derived (i.e., at a terminal branch of the phylogeny). In addition, we only counted those terminal branch haplotypes that were part of tip clades because we are interested in identifying mutations that could have been caused by the relatively recent exposure to local contaminants.

Since the sequence divergences observed in our study reflect intraspecific variation, and recombination in mtDNA is rare, we employed statistical parsimony analysis (Templeton et al. 1992) using the program TCS (Clement et al. 2000) instead of the neighbor-joining algorithm employed by Chen and Hebert (1999b). The program TCS uses the cladistic approach by Templeton et al. (1992) to create intraspecific gene genealogies by focusing on the similarities among haplotypes that differ minimally (Posada and Crandall 2001), such as by a few variable sites. We created separate nested genealogy networks for fragment 1 and fragment 2 to investigate the phylogenetic relationship and TBH status of haplotypes for each gene region. Based on this nested genealogical network, haplotypes that differed by a single mutational step and occupied terminal branch positions in tip clades were considered of recent origin and given TBH status. If both fragments 1 and 2 had shown a single base pair change in the same individual, then the individual would have been excluded from classification as a TBH for either fragment; however, no such case occurred in this analysis. We tested the null hypothesis that the incidence of terminal branch haplotypes was similar among the paired contaminated and reference sites by using the G-tests of independence (Sokal and Rohlf 1995) on the combined data for fragment 1 and 2.

RESULTS

Metals concentrations in soil and liver tissue

Soil concentrations of the metals for each site are presented in Table 1. Significantly higher levels of heavy metals were found at the DePue site when compared to the reference site at Goose Lake for each metal tested (Cd, Cu, Hg, Pb, Zn). The southern Illinois site P1 showed significantly higher levels of Cd and Hg when compared to the reference site CS. Tissue concentrations at DePue showed significantly higher concentrations for Cd and Pb when compared with the reference site CS; mercury was slightly higher at CS compared to DePue (Table 2). Tissue samples from the reference and contaminated sites in southern Illinois did not differ for the analyzed metals Cr, Pb and Hg (Table 2).

Sequence Variation in mtDNA Fragments 1 and 2

Sequencing revealed 21 variable sites in fragment 1, and 42 variable sites in fragment 2. Fragment 1 contained a greater number of genetic rearrangements, as it contained an insertion, a single nucleotide and a dinucleotide deletion (haplotypes L, N and O, respectively) compared to only one dinucleotide insertion in fragment 2 (haplotypes j, o and p). All other variation was due to nucleotide substitution with a transition to transversion

bias of 9.5:1 for fragment 1 and ~6:1 for fragment 2. Polymorphic sites were more prevalent in fragment 2, which contained 66.6% of the variable sites, and represents the more variable part of the already highly variable D-loop region.

Haplotype frequencies and their distribution

Fifteen haplotypes for white-footed mice were identified for mtDNA fragment 1 (384 bp), and 26 haplotypes for mtDNA fragment 2 (427 bp) out of a total of 46 sequences (Tables 3 and 4). The number of fragment 1 haplotypes was similar at the DePue, CS and P1 sites (Table 3). Goose Lake was the exception with only a third as many. The number of haplotypes for fragment 2 ranged from four to eleven across sites, and Goose Lake again contained the fewest haplotypes (Table 4).

The distribution of haplotypes differed greatly between the two fragments (Tables 3 and 4). For fragment 1, three of four study sites had one dominant haplotypes, representing ~35% or more of the haplotypes present. Fragment 2 showed a larger number of haplotypes, but only Goose Lake contained dominant haplotypes. In addition, haplotypes tended to be private. Over 75% of the haplotypes were present at only one site for both fragments (Tables 3 and 4). Most private haplotypes were present at relatively low frequency (<15%), but a few, such as haplotype aa, were dominant. Finally, there was striking geographic clustering among haplotypes; the distributions of northern and southern haplotypes did not overlap except for haplotype I, which was shared among sites CS, DePue and Goose Lake (Table 3 and 4).

To assess whether our results represented the majority of haplotypes present at each site, the cumulative number of novel haplotypes was plotted against the cumulative number of mice throughout the trapping season. Haplotype number for both fragments 1 and 2 approached saturation with increasing sampling effort implying that few novel haplotypes remained unsampled at each site.

Identification and Quantification of Terminal Branch Haplotypes

The TCS nested network analysis for fragment 1 revealed that only haplotype ‘G’ showed a one-step nucleotide difference from other haplotypes and occupied a terminal position in a tip clade, defining it as a terminal branch haplotype (Figure 2). The TBH was only present at the contaminated site P1, and neither the reference sites (Goose Lake and CS), nor the second contaminated site at DePue showed this recently derived G haplotype (Tables 3 and 5). Fragment 2 yielded haplotypes ‘p’ and ‘k’ that could be classified as TBHs (Figure 3), and they were both found only at the southern Illinois sites (Table 4 and 5). Specifically, haplotype k was only found at the reference site CS, whereas haplotype p was only found at the contaminated site P1 (Table 4 and 5).

Several measures of TBHs, defined by Chen and Hebert (1999b), were utilized to reflect the occurrence of TBHs among the sites (Table 5). The frequency of TBHs per site is indicated by ITBH, or the number of individuals that carry a terminal branch haplotype. Consequently, the Terminal Branch Frequency (TBF) is the ratio of the number of individuals with TBHs (ITBHs) to the sample size (n), and it indicates the spread of the terminal branch haplotypes within a site. The terminal branch index (TBI) is the ratio of TBHs to the sample size (n) and gives a measure of the number of newly arisen variants per site standardized by the sampling effort. Lastly, the number of TBHs compared to the

total haplotypes (TBH/H) measures the frequency of the most recently derived haplotypes among all haplotypes per site, hence is the most relevant measure of mutation rates per site for our study. As the contaminated site at DePue and the Goose Lake reference site showed no terminal branch haplotypes, all measures of recently derived mutations at these two sites were zero. In the south, we documented twice as many TBHs at the contaminated site P1 and as compared to the reference site CS. The ratio of TBH/H, which we use as estimates of recently derived mutations, however, were not significantly elevated at P1 when compared to CS (Table 5).

DISCUSSION

Chen and Hebert (1999b) reconstructed mtDNA genealogies to differentiate novel haplotypes (i.e., terminal branch haplotypes) from historical polymorphisms in natural fish populations of brown bullhead from Lake Ontario. Such discrimination of recently derived mutations from ancestral genetic variation is essential to establish probable cause for increased mutation rates, especially if recent exposure to environmental contaminants is the suspected reason for mutagenesis. By quantifying the proportion of novel haplotypes rather than simply summarizing overall nucleotide diversity, terminal branch haplotype (TBH) analysis provides one of the best tools to estimate mtDNA germline mutations in wildlife that was recently exposed to environmental mutagens.

In our study, we applied TBH analysis of mtDNA sequences to investigate the mutational effects of heavy metals exposure associated with Illinois Superfund sites in natural populations of white-footed mice. We followed Chen and Hebert's approach of combining DNA sequencing and phylogenetic analysis, but instead of applying Neighbor-Joining methods, we used statistical parsimony (Clement et al. 2000; Templeton et al. 1992), which is more appropriate for intraspecific analyses (Clement et al. 2000) and we only counted TBHs that were part of tip clades. For fragment 1 and fragment 2 of the D-loop region, we documented a total of 15 and 26 haplotypes, respectively. We did find a striking geographic north-south split in the distribution of those haplotypes, which most likely reflects independent evolutionary histories of the northern and southern mouse populations in Illinois. Consistent with the rarity of mutational events, few terminal branch haplotypes were found across our study sites, and the percentage of TBHs was not significantly influenced by contaminant exposure for either fragment.

The fact that novel haplotypes (TBHs) are not significantly higher at contaminated sites could be due to the small sample sizes. However, examination of the relationship between sampling effort and haplotype number indicated that few new haplotypes were added with increasing trapping effort. This result suggests that few haplotypes remained undetected within sites and the cumulative frequency of haplotypes per site approached saturation. Therefore, lack of resolution or misrepresentation of TBH incidence rate due to small sample sizes are unlikely explanations for the similarity in unique haplotypes across sites. The latter conclusion is supported by the fact that Chen and Hebert (1999b) could not detect differences in TBHs between their contaminated and reference sites despite very substantial sample sizes. They determined instead that demographic factors, such as population bottlenecks might have resulted in a significant difference of TBI across aquatic habitats. Habitat differences between sample locations were minimized in

our study, and preliminary population analyses suggest no major demographic differences between our sites.

An alternative explanation could be that the bioavailability and bioaccumulation of heavy metals at our study sites were insufficient to cause increased mutagenesis in mitochondrial DNA. Bio-availabilty and accumulation may vary across species, and genotoxicity of contaminants might be lower for mitochondrial than nuclear DNA (Johnson et al. 1999). For example, PCB, PAH and metals contamination of Hamilton Harbour was associated with increased mutations in nuclear minisatellite DNA of birds and mammals but not mitochondrial DNA of brown bullhead (Chen and Hebert 1999b; Somers et al. 2002; Yauk and Quinn 1996). It is difficult to ascertain the exact threshold level of heavy metals that would cause mutations in nuclear or mtDNA, since most previous wildlife studies on the accumulation and acute toxicity of metal contaminants did not quantify genetic damage (Ma 1989; Laurinolli and Bendell-Young 1996; Pascoe et al. 1994; Winder and Bonin 1993). Few studies have performed concurrent metals uptake and mutagenic analyses in the field (Peles and Barrett 1997; Tull-Singleton et al. 1994), hence the implication of heavy metals as genotoxins, even at low levels, is primarily based on lab studies (Ariza and Williams 1996; Codina et al. 1995). Some lab studies have examined the genotoxicity threshold of heavy metals (Codina et al. 1995) and mutagenic chemicals (Ogheri et al. 1995), and a few experiments have reported positive dose-response curves between heavy metal concentration and mutagenic effect (Ariza and Williams 1996; Codina et al. 1995). However, these types of studies are in the minority. It would be beneficial to the wildlife biologist if laboratory studies of mutagenic agents using animal models published not only the *dosage of chemicals applied*, but also the resulting *actual tissue levels* of chemicals. The latter is essential to draw conclusions about biologically relevant threshold levels of contaminants in natural populations where knowledge of exposure has to be inferred from soil and tissue analyses. Also, the fact that lab studies often utilize cell culture or microbial assays (Ariza and Williams 1996; Codina et al. 1995; Moore et al. 1997; Ogheri et al. 1995), and test a single metal or metal compound to determine genotoxicity (Ariza and Williams 1996; Codina et al. 1995; Winder and Bonin 1993), makes it difficult to directly extrapolate lab results to wildlife, which is often exposed to complex mixtures of metals (Chen and Hebert 1999b; Laurinolli and Bendell-Young 1996; Pascoe 1994; Peles and Barrett 1997; Somers et al. 2002; Yauk and Quinn 1996).

In summary, we successfully applied terminal branch haplotype analysis to quantify recently derived mtDNA mutations associated with heavy metal contaminated Superfund Sites and reference sites in Illinois. Sequence analysis of the mtDNA D-loop region revealed a clear geographic split in haplotype distribution, however, contaminant exposure was not associated with increased mtDNA mutation rates as measured by percentage of TBHs. We recommend that future genotoxicology studies on natural populations provide estimates of bioavailabilty and bioaccumulation of contaminants to establish a data base on critical threshold levels of contaminant exposure.

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Figure 1. Approximate location and distances between northern and southern Illinois study sites.

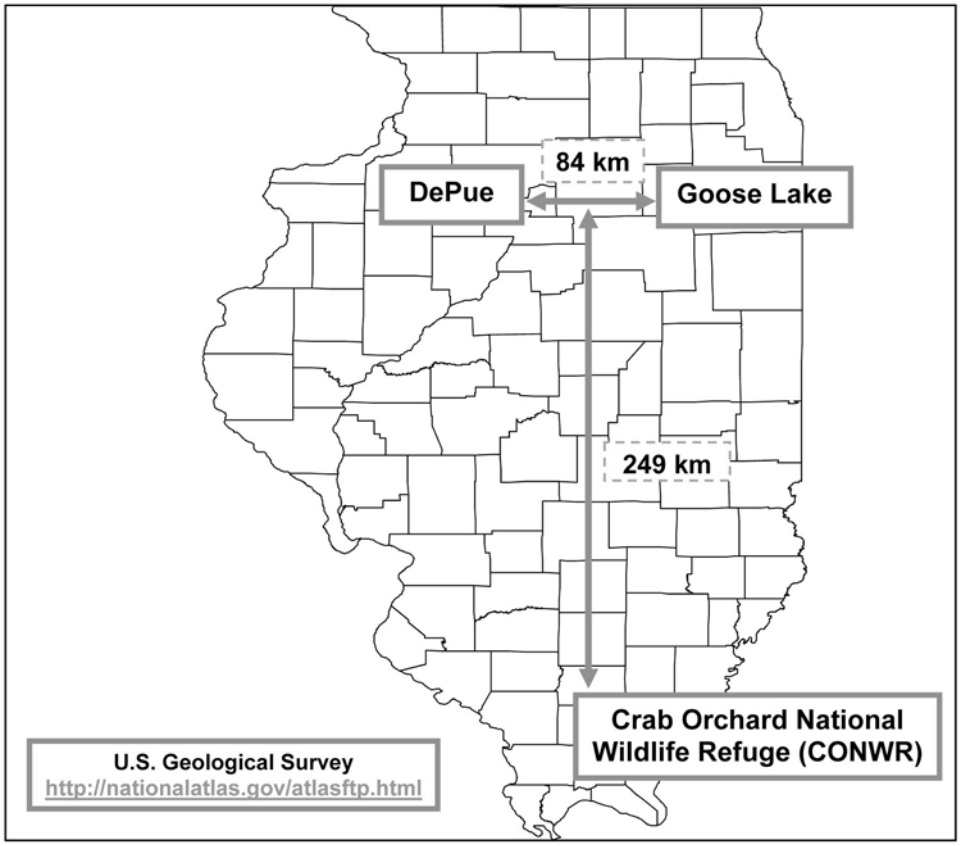

Figure 2. Nested network for fragment 1 (384 bp) haplotypes. Branches indicate 1 mutational step, missing haplotypes are indicated by 'o', haplotypes are indicated by labeled ovals, oval size does not indicate the frequency of individuals per haplotype. Within the total cladogram, haplotypes are equivalent to zero-step clades, clades at one level are clustered into higher level clades, interior clades are connected to more than one clade at the same hierarchical level, tip clades are connected to only one other clade. Haplotypes identified as TBH (i.e., recently derived mutations) had to be part of a tip clade and fulfill criteria identified in text.

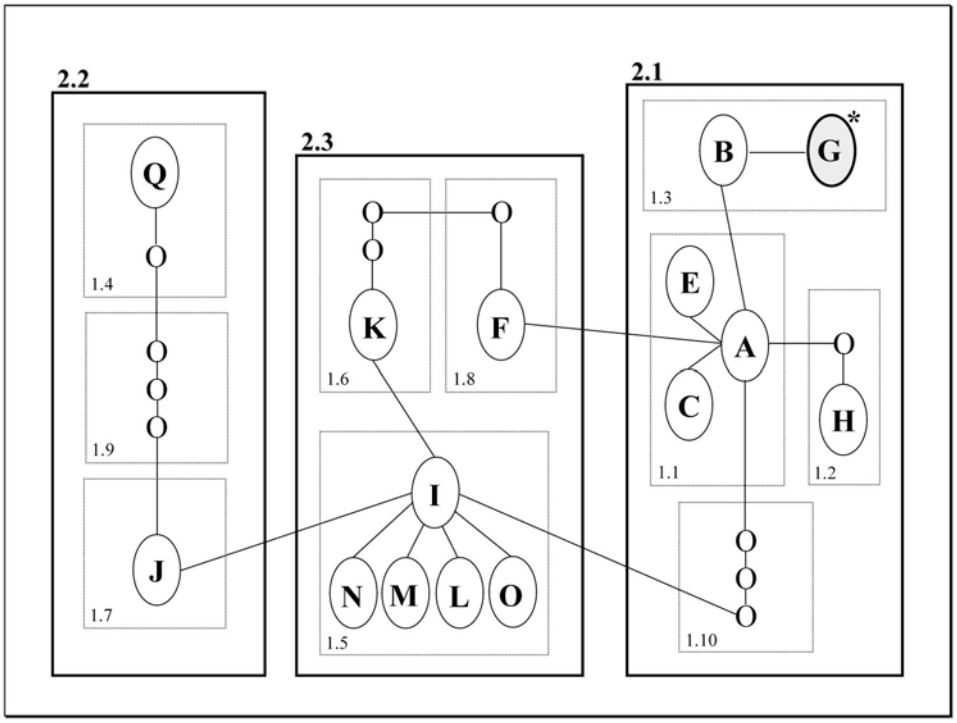

Figure 3. Nested network for fragment 2 (427 bp) haplotypes. Haplotypes identified as TBH had to be part of a tip clade and fulfill criteria identified in text (notation as above). Haplotype ‘s’ was excluded from the network as it exceeded the number of maximal mutational connections justified by ‘parsimony’ criterion.

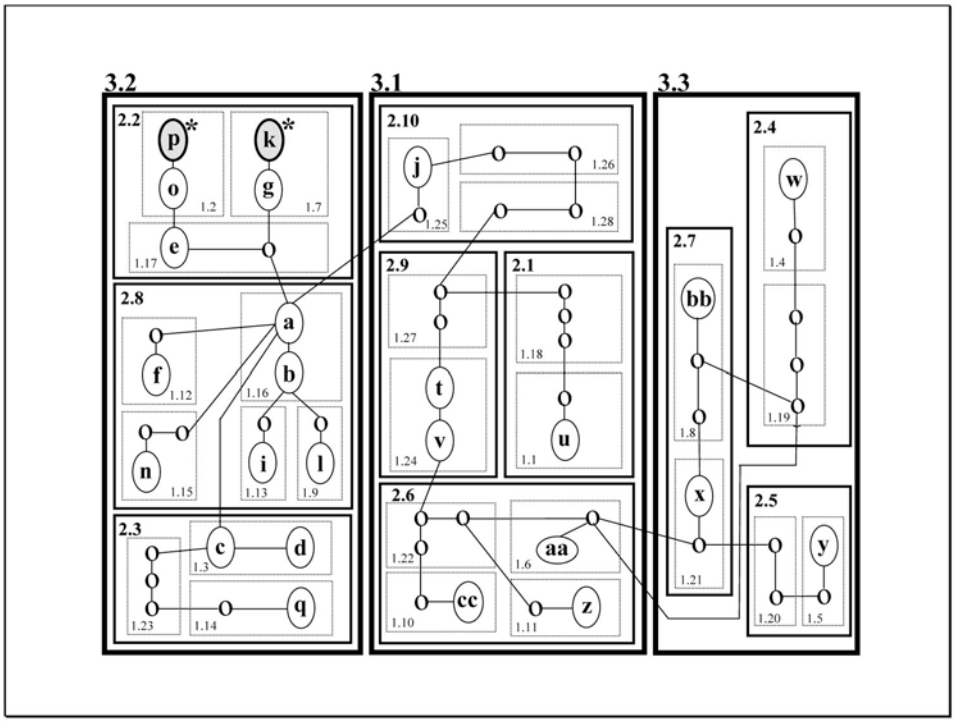

Table 1. Heavy metal concentration of soil collected at the contaminated and reference sites. The dashed line separates the northern and southern sites. An asterisk next to contaminated sites indicates the probability that values differ from each other by chance based on non-parametric statistical test (see text for details).

Site (n)	Type	Units	Statistic	Chromium	Copper	Zinc	Cadmium	Lead	Mercury
P1(2)	soil	mg/kg	MEAN:	31.95 NS	ND	ND	1.15*	26.55 NS	0.11*
			SE:	2.05	ND	ND	0.05	4.95	0.01
			RANGE:	29.9–34.0	ND	ND	1.1-1.2	21.6-31.5	0.1-0.12
CS(10)	soil	mg/kg	MEAN:	22.69	ND	ND	0.12	19.24	0.045
			SE:	0.63	ND	ND	0.01	0.95	0.003
			RANGE:	19–25	ND	ND	<0.1-0.19	16-23	0.027-0.064
DePue (10)	soil	mg/kg	MEAN:	ND	42.9*	2497.31*	38.6*	194.77*	0.046*
			SE:	ND	9.34	828.91	11.34	54.03	0.007
			RANGE:	ND	13 - 98	527-8020	4.7-106	41-466	0.032-0.089
Goose Lake (10)	soil	mg/kg	MEAN:	ND	14.53	59.01	0.32	22.93	0.028
			SE:	ND	0.49	2	0.02	0.73	0.03
			RANGE:	ND	13 - 18	47-67	0.24-0.50	21-28	0.014-0.40

ND = not done; NS = Not significant; * $P < 0.05$

Table 2. Heavy metal concentration of tissue from mice collected at the contaminated and reference sites. The dashed line separates the northern and southern sites; tissue from Goose Lake was not available for this analysis. An asterisk next to contaminated sites indicates the probability that values differ from each other by chance based on non-parametric statistical test (see text for details).

Site (n)	Type	Units	Statistic	Chromium	Copper	Zinc	Cadmium	Lead	Mercury
P1(5)	liver	mg/kg	MEAN:	0.06 NS	ND	ND	ND	0.22 NS	0.0074 NS
			SE:	0.01	ND	ND	ND	0.07	0.0003
			RANGE:	<0.05-0.08	ND	ND	ND	<0.05-0.4	0.007-0.009
CS(5)	liver	mg/kg	MEAN:	0.06	5.58	23.8	0.04	<0.05	0.0072
			SE:	0.004	0.34	0.92	0.01	0	0.0007
			RANGE:	<0.05-0.7	4.6-6.4	22-27	0.02-0.07	<0.05	0.005-0.009
DePue(5)	liver	mg/kg	MEAN:	ND	6.22 NS	27 NS	0.93*	0.24*	0.0022*
			SE:	ND	0.35	1.7	0.38	0.08	0.0002
			RANGE:	ND	5.4-7.1	25-33	0.3-1.7	<0.05-0.4	0.001-0.003

ND = not done; NS = Not significant; * $P < 0.05$

Table 3. The frequency and distribution of 17 mtDNA fragment 1 (384 bp) haplotypes in white-footed mice. Asterisks indicate Terminal Branch Haplotypes based on criteria outlined in text.

	Study Sites			
Haplotypes	P1	CS	DP	GL
A	50.0	28.6	0.0	0.0
B	7.1	21.4	0.0	0.0
C	7.1	0.0	0.0	0.0
E	0.0	7.1	0.0	0.0
F	14.3	0.0	0.0	0.0
G*	14.3	0.0	0.0	0.0
H	0.0	28.6	0.0	0.0
I	0.0	7.1	30.0	62.5
J	0.0	0.0	10.0	0.0
K	0.0	0.0	10.0	0.0
L	0.0	0.0	20.0	0.0
M	0.0	0.0	0.0	37.5
N	7.1	0.0	0.0	0.0
O	0.0	0.0	30.0	0.0
Q	0.0	7.1	0.0	0.0

Table 4. The frequency and distribution of 29 mtDNA fragment 2 (427 bp) haplotypes in white-footed mice. Asterisks indicate Terminal Branch Haplotypes (TBH), which are considered equivalent to recently derived mutations based on criteria outlined in text.

	Study Sites			
Haplotypes	P1	CS	DP	GL
a	0.0	28.6	0.0	0.0
b	7.1	0.0	0.0	0.0
c	7.1	0.0	0.0	0.0
d	14.3	0.0	0.0	0.0
e	14.3	14.3	0.0	0.0
f	14.3	0.0	0.0	0.0
g	7.1	7.1	0.0	0.0
i	0.0	7.1	0.0	0.0
j	0.0	7.1	0.0	0.0
k*	0.0	14.3	0.0	0.0
l	0.0	7.1	0.0	0.0
n	7.1	0.0	0.0	0.0
o	7.1	0.0	0.0	0.0
p*	7.1	0.0	0.0	0.0
q	7.1	0.0	0.0	0.0
s	0.0	7.1	0.0	0.0
t	0.0	0.0	0.0	12.5
u	0.0	7.1	0.0	0.0
v	0.0	0.0	20.0	12.5
w	7.1	0.0	0.0	0.0
x	0.0	0.0	10.0	0.0
y	0.0	0.0	30.0	0.0
z	0.0	0.0	20.0	37.5
aa	0.0	0.0	0.0	37.5
bb	0.0	0.0	10.0	0.0
cc	0.0	0.0	10.0	0.0

Table 5. The terminal branch-haplotype ratio, the terminal branch index (TBI) and terminal branch frequency (TBF) in white-footed mice for fragment 1 (384 bp) and fragment 2 (427 bp) combined. H: Haplotype, ITBH: Individuals with TBH. Terminal Branch Haplotypes are considered equivalent to recently derived mutations based on criteria outlined in text.

Region	Site	Sample (*n*)	No. of TBH	No. of H	No. of ITBH	[a]Ratio of TBH/H	[b]TBI (TBH/n)	TBF (ITBH/n)
North	DePue	10	0	5	0	0.0%	0.0%	0.0%
	Goose Lake	8	0	2	0	0.0%	0.0%	0.0%
South	P1	14	2	17	3	11.8%	14.3%	21.4%
	CS	14	1	15	2	6.7%	7.1%	14.3%

[a,b] not significantly different when comparing reference and contaminated sites