

IDENTIFYING VOUCHER SPECIMENS INVOLVING RISK: SHREWS FROM CHORNOBYL, UKRAINE

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Specimens may pose a risk to personnel handling them such as the radioactive fauna collected from contaminated areas in Chernobyl. To minimize radiation exposure and to evaluate an inexpensive molecular tool in identifying species of shrew, we analyzed DNA sequences from the cytochrome-*b* gene. Specimens were visually identified as *Sorex araneus*, *S. minutus*, and *Neomys fodiens*, and morphological variants were noted that could represent additional species. Cytochrome-*b* sequences indicated the apparent variants represented intraspecific polymorphisms. This approach substantially reduced exposure to radioactivity present in the archived material. Excluding salaries and cost of equipment, the analytical cost per specimen was US\$9.00. The benefit and accuracy in identifying specimens this way is justified based on the reduction of risk these samples may pose.

Key words: Chernobyl, cytochrome *b*, *Neomys*, radiation, risk, shrews, *Sorex*

Proper identification of species is required in cataloging biodiversity and is essential in studies investigating microevolutionary (genetic, ecological, and life-history traits and ecotoxicology) or macroevolutionary (systematics) characteristics. These include studies in which specimens pose a risk to scientists collecting, preparing, and identifying them. For example, a health risk may exist from specimens inhabiting sites contaminated with physical or chemical pollutants (or both) or where they serve as a vector for human disease. Methods that reduce risk and still allow for safe curation and use of voucher specimens will be valuable.

In this study, we documented biodiversity of shrews in the most radioactive areas contaminated as a result of the accident at Chernobyl, Ukraine. This is an appropriate

model for testing alternative methods of identifying voucher specimens because Soricidae is one of the most widely distributed and diverse mammalian families (23 genera, 312 species—Hutterer 1993). Additionally, many species of shrews are not well defined morphologically, which makes field identification problematic. In part, this is due to their body plan, which is rather uniform across species (Fumagalli et al. 1999). Political factors, such as the restriction of scientific exchange resulting from language barriers, lack of access to collection localities across political boundaries, and limited exchange of museum specimens, also contribute to difficulties in classifying fauna from Chernobyl.

In our ecotoxicological research in the Chernobyl region, we applied a combination of emerging and standard methods to estimate the number of species of shrews and relative abundance of each species in

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the region. Molecular data were substituted for relatively lengthy, standard morphological analyses, in an effort to minimize radiation exposure of museum personnel while using the classical approach. At least 3 species, *Sorex araneus*, *S. minutus*, and *Neomys fodiens* are known to occur in the Chernobyl region. However, apparent external morphological variants (e.g., pelage color and pattern, general size, pinnae characteristics) have been observed during our sampling of the region. An alternative to the null hypothesis of only 3 species present at Chernobyl is that these morphological variants represent additional species. Therefore, we chose to examine variability in mitochondrial DNA cytochrome-*b* (*Cytb*) sequences to estimate the phylogenetic patterns present within our shrew specimens. It was critical to this approach that the region of DNA chosen exhibits a level of variation suitable for detecting congeneric relationships among species. The *Cytb* gene has demonstrated the capacity to discriminate between congeneric species in numerous mammalian groups including shrews (Avice and Walker 1999; Bradley and Baker 2001; Fumagalli et al. 1999; Johns and Avice 1998).

MATERIALS AND METHODS

Specimens ($n = 126$) were prepared in a field laboratory following museum standards as skin and skeleton, alcoholic, or skull-only voucher specimens. Tissues archived include heart, liver, kidney, bone, and muscle. After transport to the permanent museum collection at the Natural Science Research Laboratory, Texas Tech University, Lubbock, Texas, specimens were isolated from the primary collection area to minimize radioactive exposure of museum staff. Genomic DNA was isolated using standard procedures (Longmire et al. 1997). A Packard Biosciences Cobra Quantum gamma counter (Packard Biosciences/Perkin-Elmer Life Sciences, Boston, Massachusetts) calibrated using a $^{137}\text{cesium}$ source was used to measure residual radioactivity in isolated DNA. Radioactivity in DNA was at background levels and well below minimal

limits established by radiation safety standards, thereby eliminating risk to laboratory personnel.

The entire *Cytb* (1,140 base pairs [bp]) was amplified from approximately 200 ng of genomic DNA using the polymerase chain reaction (PCR). The primers L14724 and H15915 were used in the PCR (Irwin et al. 1991). Thermal cycling parameters for the PCR consisted of 35 cycles of denaturation at 95°C for 50 s, primer annealing at 50°C for 45 s, and strand extension at 72°C for 2 min followed by a 7-min final extension at 72°C. PCR products were purified using the QIAGEN PCR purification kit following the manufacturer's recommendations (QIAGEN Corporation, Valencia, California). Both light and heavy strands were then cycle sequenced for a subset of apparent morphological variants ($n = 28$) with 2 internal primers, LGL765 (Bickham et al. 1995) and H15149 (Irwin et al. 1991), using the ABI PRISM® dRhodamine or ABI PRISM® BigDye™ dye-terminator chemistry (Applied Biosystems Incorporated, Foster City, California). These 2 primers span the first 400 bp of *Cytb*. The remaining shrews ($n = 98$) were cycle sequenced in 1 direction using the primer LGL765. Raw DNA chromatograms were generated using capillary electrophoresis and a fast sequencing protocol on a genetic analyzer (ABI PRISM® 310) according to the manufacturer's recommendations (Applied Biosystems Incorporated). Chromatograms and DNA sequences were then proofed and aligned using the Sequencher ver 3.1 software (Gene Codes Corporation, Ann Arbor, Michigan).

Phylogenetic analyses were conducted using MEGA version 2.1 (Kumar et al. 2001). The Tamura-Nei distance model was used to estimate genetic distances among specimens and groups of specimens (Tamura and Nei 1993). The minimum-evolution method was used to generate phylograms based on estimates of genetic distance (Rzhetsky and Nei 1992).

To provide a larger geographic context for systematic identification of voucher specimens, we included, in a larger analysis, published DNA sequences for 7 species of soricid, including *S. araneus*, *S. minutus*, and *Neomys fodiens*, obtained from GenBank (National Center for Biotechnology Information, Bethesda, Maryland). The bootstrap method (Felsenstein 1985) was applied to assess node consistency in all analyses.

RESULTS

Ukrainian samples.—From 28 specimens, we used 272 bp (5' portion) of *Cytb* representing 90 codons. A total of 15 unique haplotypes were observed with Tamura–Nei distance values of 0.0–24.7%. Two hundred five characters (75%) were constant among all taxa, 3 characters (1%) were unique to a single individual, and 64 characters (24%) were shared among at least 2 individuals but not among all taxa analyzed.

Three phylogroups were formed with high bootstrap support (>95%). Seven unique haplotypes were found within phylogroup A_{UA} (*n* = 18; subscript UA refers to specimens from Ukraine), and mean genetic variation within A_{UA} was 0.3% ± 0.1 SE (Fig. 1). Four unique haplotypes were found within phylogroup B_{UA} (*n* = 5), and mean genetic variation within B_{UA} was 0.7 ± 0.3% (Fig. 1). Four unique haplotypes were found within phylogroup C_{UA} (*n* = 5), and mean genetic variation within C_{UA} was 0.9 ± 0.4% (Fig. 1). The average distance between the phylogroups A_{UA}, B_{UA}, and C_{UA} was 9.1 ± 1.9% (from A_{UA} to B_{UA}), 24.7 ± 3.7% (from A_{UA} to C_{UA}), and 23.7 ± 3.6% (from B_{UA} to C_{UA}).

All samples.—From 35 specimens, we used 272 bp (5' portion) of *Cytb* including samples from Ukraine and specimens of *N. fodiens* (*n* = 1), *S. minutus* (*n* = 1), *S. araneus* (*n* = 1), *S. samniticus* (*n* = 1), *S. tundrensi* (*n* = 1), *S. corona* (*n* = 1), and *S. granarius* (*n* = 2) from GenBank (National Center for Biotechnology Information). Seventeen unique haplotypes were observed with Tamura–Nei distance values of 0.0–24.6%. Two hundred one characters (74%) were constant among all taxa, 3 characters (1%) were unique to a single individual, and 68 characters (25%) were shared among at least 2 individuals but not among all taxa analyzed.

Three phylogroups were again formed with moderate bootstrap support (>67%; Fig. 2). Seven unique haplotypes were

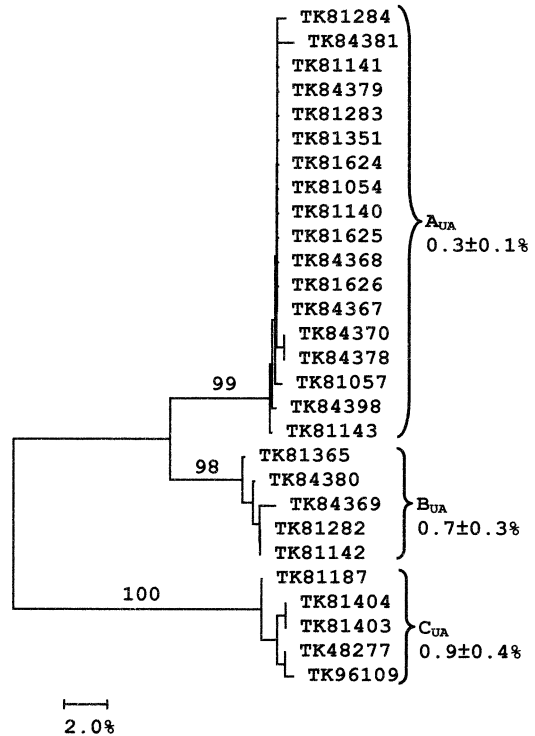


FIG. 1.—Minimum-evolution phylogram depicting genetic (400 bp of *Cytb*) relationships among shrew specimens collected from Chernobyl. Taxon codes (TK numbers) correspond to voucher specimens deposited in the Natural Science Research Laboratory, Texas Tech University. All taxa were collected in the Chernobyl region of Ukraine (UA used as subscript), and letter designations group clades with high bootstrap support (values placed on branches). Percentage values represent mean genetic variation and SEs within designated clades; scale is provided below phylogram.

found within phylogroup A_{ALL} (*n* = 19; subscript ALL refers to all taxa assessed), and mean genetic variation within A_{ALL} was 0.3 ± 0.1% (Fig. 2). The single representative of *S. araneus* from GenBank fell within this phylogroup. Five unique haplotypes were found within phylogroup B_{ALL} (*n* = 6), and mean genetic variation within B_{ALL} was 1.1 ± 0.4% (Fig. 2). The single representative of *S. minutus* from GenBank fell within this phylogroup. Five unique haplotypes were found within phylogroup

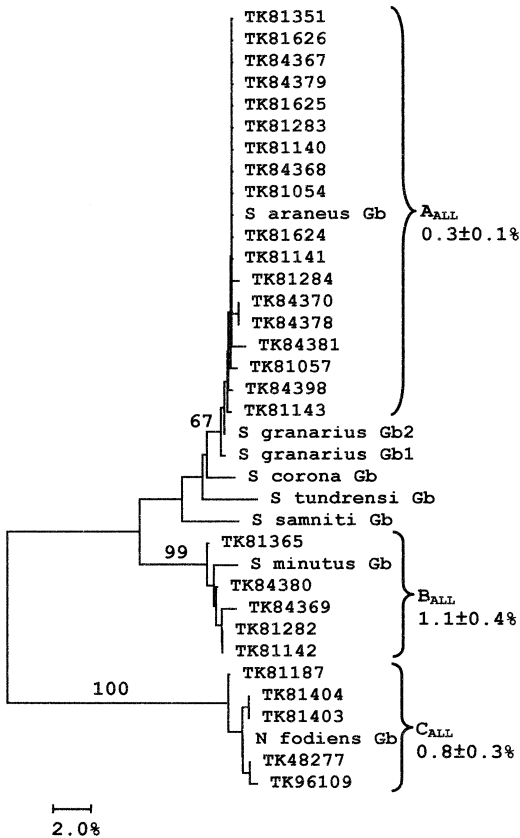


FIG. 2.—Minimum-evolution phylogram depicting genetic (400 bp of *Cytb*) relationships among shrew specimens collected from Chernobyl. Taxon codes (TK numbers) correspond to voucher specimens deposited in the Natural Science Research Laboratory, Texas Tech University. Letter designations grouping clades follow those used in Fig. 1 except for the subscript. Subscript ALL denotes all taxa for which 400 bp of sequence data were available for analysis, including those obtained from GenBank. GenBank individuals are identified by their binomials and Gb. Values along branches are bootstrap estimates. Percentage values represent mean genetic variation and SEs within designated clades; scale is provided below phylogram.

C_{ALL} ($n = 6$), and mean genetic variation within C_{ALL} was $0.8 \pm 0.3\%$ (Fig. 2). The single representative of *N. fodiens* from GenBank fell within this phylogroup. The average distance between the phylogroups A_{ALL}, B_{ALL}, and C_{ALL} was $9.3 \pm 1.9\%$ (from

A_{ALL} to B_{ALL}), $24.6 \pm 3.7\%$ (from A_{ALL} to C_{ALL}), and $23.9 \pm 3.4\%$ (from B_{ALL} to C_{ALL}). The GenBank specimens of *S. granarius* fell within phylogroup A_{ALL} in a condensed tree (not presented); however, in the phylogram, these 2 specimens are peripheral to the specimens allied with *S. araneus* (Fig. 2). The remaining species were intermediate between phylogroup A_{ALL} and B_{ALL}.

Each unique *Cytb* DNA sequence was submitted to GenBank according to our molecular taxonomic assignment. Accession numbers are AF445032–AF445037 for *S. araneus*, AF445038–AF445041 for *S. minutus*, and AF445028–AF445031 for *N. fodiens*.

Finally, a minimum of 240 bp of light-strand *Cytb* data were obtained for each remaining specimen ($n = 98$). Taxonomic affinities based on genetic analyses indicated that all these specimens were most closely related to *S. araneus* (phylogroup A in both Figs. 1 and 2; data not shown). The overall average distance within this assemblage was $0.2 \pm 0.1\%$.

DISCUSSION

In the field, investigators identified at least 3 species of shrew in the Chernobyl environment. Additionally, variation in pelage color, size of pinnae, and extent of hair on the tail suggested that there were >3 species of shrews. However, only 3 major subdivisions could be substantiated in the genetic analyses, and these corresponded to the 3 species identified by external morphology. Because apparent variation in external morphology was not reflected in the *Cytb* analyses, we conclude that only 3 species of shrews (*S. araneus*, *S. minutus*, and *N. fodiens*) are present in our Chernobyl collection. Phylogroup A comprises specimens we classify as *S. araneus*, phylogroup B comprises specimens we classify as *S. minutus*, and phylogroup C comprises specimens we classify as *N. fodiens* (Figs. 1 and 2). These assignments are consistent with published data. We, therefore, conclude that

these morphological variants are simply polymorphisms within *S. araneus* inhabiting the Chernobyl region. Voucher specimens were accessioned in the permanent, isolated collection according to taxonomic assignment using field identifications and accompanying molecular analyses (Natural Science Research Laboratory, Texas Tech University). In total, we have cataloged 5 specimens of *N. fodiens*, 6 specimens of *S. minutus*, and 115 specimens of *S. araneus* using this molecular approach.

We believed that it was justifiable to examine all specimens of shrews using the molecular approach because of the potential risk involved in handling them in the classical curatorial manner. Chesser et al. (2000) concluded that shrews have an average internal radiocesium activity of approximately 632.3 Bq/g of muscle tissue. According to Turner (1995), treating each specimen as a point source and assuming an average of 5 g of total muscle mass per specimen would yield an estimated dose rate of approximately 4.4×10^{-4} mrem/hr at a distance of 0.25 m. If unshielded museum personnel performing standard morphological analyses spend 8 h/day identifying these specimens (10 specimens/day), they would incur an annual dose of approximately 9.1 mrem. Current regulatory statutes mandate a maximum annual, occupational total effective dose equivalent of 5 rem (Texas Regulations for Control of Radiation, in litt.). Assuming that the personnel are involved in collecting specimens, an additional 600 mrem will be absorbed from the Chernobyl environment, yielding a total annual dose of 0.61 rem. For shrews, this is well below occupational limits. However, some species of rodent such as the bank vole, *Clethrionomys glareolus*, and possibly other species of rodent or small carnivore, have substantially higher levels of radioactivity (average of 5,062.2 Bq/g) and are larger (approximately 20 g/bank vole). The annual estimated dose from examining specimens such as these will approach 15 mrem. If only the most radioactive speci-

mens are collected and analyzed (73,090 Bq/g), the annual dose approaches 0.82 rem. To minimize the fraction of the total allowable dose, we developed and applied a molecular substitute to traditional morphological analysis. This molecular approach is meant to minimize radiation exposure; however, analyzing the radioactive, archived voucher specimens using traditional morphological analysis is not necessarily prohibited. Personnel will have to be properly trained in techniques and procedures designed to minimize radiation exposure, and criteria for monitoring absorbed dose, limiting handling time, and shielding will have to be applied. However, this increases costs associated with standard analyses.

This study documents that it may be economically feasible to use our molecular approach to examine levels of genetic variability and taxonomic affinity for samples of other individuals. Based on current costs incurred in obtaining a single DNA sequence (approximately 400 bp in 1 direction) using fluorescent technology, a reasonable estimate of this cost is approximately US\$9.00. This is an estimate of the cost of consumable materials alone and does not include salaries or cost of essential equipment. After molecular assignment, a subset of a field sample can provide baseline morphological characteristics to assign taxonomic designations to the remaining specimens in the collection.

The benefit of archiving voucher specimens is greater than any costs associated with any potential risk. Curating high-risk specimens will considerably increase the financial burden on accredited institutions, but the benefit to society in terms of the knowledge these specimens can provide justifies their worth. Methods, such as the molecular approach used in this study, will serve to reduce potential risk associated with handling biological materials in some cases. It is not our intent to provide a rigid framework for identifying any specimens that pose a risk to curatorial or research per-

sonnel (or both). This is because perceived and potential risks are constantly evolving as well (e.g., tissues may be a source of risk rather than skeletons). Instead, we intend to provide a specific example that allows for systematic affiliations to be estimated in specimens that pose a radiation risk. A commitment must be made by accredited natural history collections and the public to maintain archived material for future research, and this includes applying methods that eliminate or reduce risk to personnel handling biological specimens.

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