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Identification of Skunk Species Submitted for Rabies Testing in the Desert Southwest

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ABSTRACT: Skunks usually are identified by their common name (skunk) when submitted for rabies testing. In the desert southwest (Arizona, New Mexico, Texas, USA; and northern Mexico), there are five species of skunks; four of which can occur in sympatry. To better understand the ecology of skunk rabies in these areas, it is imperative that species be properly identified. We used the displacement loop (d-loop) of the mitochondrial genome to identify to species 24 skunk brain samples submitted for rabies testing in New Mexico from 2001 to 2002. Most were identified as striped skunks (*Mephitis mephitis*), but hooded (*Mephitis macroura*) and hog-nosed (*Conepatus leuconotus*) skunks were also found.

Key words: Conepatus, Mephitidae, *Mephitis,* mitochondrial DNA, rabies, *Spilogale,* taxonomy, voucher specimens.

Skunks are one of the primary wildlife species responsible for reported cases of rabies in the United States, accounting for approximately 31% of positive animals (Krebs et al., 2002). The threat of human exposure to rabies virus from skunks directly or indirectly is a well-recognized health threat. Striped skunks (*Mephitis mephitis*) are often attracted to housing areas by the presence of pet food, water, garbage, and high populations of invertebrates in urban landscaping. Consequently, they are more likely to encounter humans and their pets.

The majority of skunk rabies cases in Arizona (Arizona Department of Health Services, Phoenix, Arizona, USA) is limited to the zone of sympatry for four skunk species. During 1999–2002, Arizona reported 11, 17, 59 (19 from Flagstaff), and 44 rabid skunks, respectively, whereas New Mexico reported three, nine, two, and three rabid skunks. The low number reported in New Mexico was due to passive surveillance only. Unfortunately, it is not known what species of skunk were positive for rabies because specimens were only identified to family (Mephitidae).

The objective of this study was to identify species of skunk submitted for rabies testing in 2000–01 from New Mexico using brain samples labeled "skunk." We compared the displacement loop (d-loop) DNA sequence of skunk samples of unknown species to known species specimens.

DNA was extracted from 24 brains (Table 1) stored in lysis buffer (Longmire et al., 1997) using a DNeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA). Polymerase chain reaction (PCR) was used to amplify the d-loop region of mitochondrial DNA using primers L16272 and H1008 and following procedures used by Dragoo et al. (2003). Cleaned PCR products were only sequenced with primer L16272 using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Inc., Foster City, California, USA). Sequences were determined by running samples on a 377 ABI Prism DNA Sequencer (Applied Biosystems, Inc.).

Additionally, 12 specimens from known skunk species (Table 1) were sequenced or obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) for the complete gene as reference samples and included Oriental stink badger (*Mydaus marchei*) as an outgroup, striped hog-nosed skunk (*Conepatus chinga*), white-backed hog-nosed skunk (*Conepatus leuconotus*), striped skunk, hooded skunk (*Mephitis macroura*), eastern spotted skunk (*Spilogale putorius*),

TABLE 1. General locality and species indentification of skunk samples tested from New Mexico. Brains from samples identified as skunk were analyzed individually from reference samples. Maximum parsimony analyses of individual brain samples resulted in single most parsimonious trees (see Fig. 1 as example). Clades into which samples were placed are identified by species. Node support is provided for placement of unknown brain samples (bootstrap and decay indices).

Specimen identification[a]	Sample label	Locality[b]	Species clade	Bootstrap[c]	Decay index[c]
SLD200101786	Skunk	Roswell	*Mephitis mephitis*	100	11
SLD200102003	Skunk	Tucumcari	*M. mephitis*	100	9
SLD200102093	Skunk	Rio Rancho	*M. mephitis*	100	9
SLD200102095	Skunk	Las Cruces	*M. mephitis*	100	11
SLD200102112	Skunk	Alamogordo	*M. mephitis*	100	12
SLD200102386	Skunk	Rio Rancho	*M. mephitis*	100	10
SLD200102388	Skunk	Las Cruces	*M. mephitis*	100	11
SLD200102390	Skunk	Alamogordo	*M. mephitis*	100	12
SLD200102644	Skunk	Clayton	*M. mephitis*	100	11
SLD200102663	Skunk	Santa Fe	*M. mephitis*	100	10
SLD200102739	Skunk	Albuquerque	*Conepatus leuconotus*	97/100[a]	3/12[a]
SLD200102802	Skunk	Aztec	*M. mephitis*	100	10
SLD200102816	Skunk	Rio Rancho	*M. mephitis*	100	9
SLD200102834	Skunk	Alamogordo	*M. mephitis*	100	10
SLD200103008	Skunk	Carlsbad	*M. mephitis*	100	12
SLD200103010	Skunk	Alamogordo	*M. mephitis*	100	12
SLD200103091	Skunk	White Sands	*M. mephitis*	100	11
SLD200103153	Skunk	Rio Rancho	*M. mephitis*	100	9
SLD200103177	Skunk	Alamogordo	*M. mephitis*	100	10
SLD200103254	Skunk	Alamogordo	*M. mephitis*	100	11
SLD200200433	Skunk	Truth or Consequences	*M. mephitis*	100	10
SLD200200763	Skunk	Alamogordo	*M. mephitis*	100	12
SLD200200811	Skunk	Lordsburg	*M. macroura*	99	7
SLD200201263	Skunk	Los Lunas	*M. mephitis*	100	12
AY159818	*Conepatus chinga*	Bolivia	*C. chinga*	100	12
AY159816	*Conepatus leuconotus*	Tamaulipas, Mexico	*C. leuconotus*	100	8
AY159817	*Conepatus leuconotus*	Junction, Texas	*C. leuconotus*	100	8
MSB92700	*Mephitis mephitis*	College Station, Texas	*M. mephitis*	100	11
MSB135534	*M. mephitis*	Fort Huachuca, Arizona	*M. mephitis*	100	11

TABLE 1. Continued.

Specimen identification[a]	Sample label	Locality[b]	Species clade	Bootstrap[c]	Decay index[c]
MSB135248	*Mephitis macroura*	Fort Huachuca, Arizona	*M. macroura*	100	5
MSB135532	*M. macroura*	Fort Huachuca, Arizona	*M. macroura*	100	5
MSB135535	*Spilogale putorius*	Bangs, Texas	*S. putorius*	100	13
TCWC43632	*S. putorious*	Eagle Lake, Texas	*S. putorius*	100	13
MSB135247	*Spilogale gracilis*	Fort Huachuca, Arizona	*S. gracilis*	99	8
MSB60873	*S. gracilis*	Chihuahua, Mexico	*S. gracilis*	99	8
CMC M2590	*Mydaus marchei*	Palawan, Philippines	*M. marchei*	Outgroup	

[a] Specimen identification number are as follows: AY number are GenBank accession numbers; CMC, Cincinnati Museum Center at the Cincinnati Museum of Natural History, Cincinnati, Ohio (USA); MSB, Division of Mammals, Museum of Southwestern Biology; SLD, Scientific Laboratory Division, New Mexico Department of Health, Albuquerque, New Mexico; TCWC, Texas Cooperative Wildlife Collections, Texas A&M University, College Station, Texas.

[b] Location of skunk samples submitted to the New Mexico Department of Public Health. Samples are from New Mexico unless otherwise indicated.

[c] Bootstrap and decay indices are for species (*Conepatus leuconotus*)/genus (*Conepatus*).

and western spotted skunk (*Spilogale gracilis*). Sequences obtained from this study were submitted to GenBank (AY587074–AY587106).

Maximum parsimony, using the exhaustive search option, was used to derive relationships from the nucleotide sequence data from the 12 known specimens using PAUP* 4.0 (Swofford, 1999). Tree length was used to determine the most parsimonious solution, and support for individual clades was evaluated using both the decay index (Bremer, 1988) using Autodecay v. 4.0.2 (Eriksson, 1998) and bootstrap resampling (Felsenstein, 1985) using 10,000 replications of random addition of taxa and tree bisection reconnection (TBR) branch swapping. This analysis produced a single most parsimonious tree (not shown). There was strong bootstrap and decay index support for nodes grouping the species as well as strong support for nodes grouping genera, whereas weaker support was found for relationships among genera.

Once relationships of known specimens were determined, unknown samples were added individually to the analyses. Because the extra skunk in each analysis precluded an exhaustive search, a heuristic search option with 100 replications of random addition of taxa and TBR branch swapping was used instead. Again, bootstrap and decay indices were obtained for each node found for the most parsimonious tree. These trees were used to identify unknown skunk brain samples to species. The species clade to which brain samples grouped determined the skunk species from which the sample was obtained.

Individual analyses of brain samples compared with reference samples also showed strong support for species and genus relationships (Table 1). When analyzed with reference specimens, 22 brain samples were identified as striped skunk, one was identified as a hog-nosed skunk, and one as a hooded skunk. These analyses generated 24 trees (one per sample; data not shown). A tree containing reference samples and three representatives of each

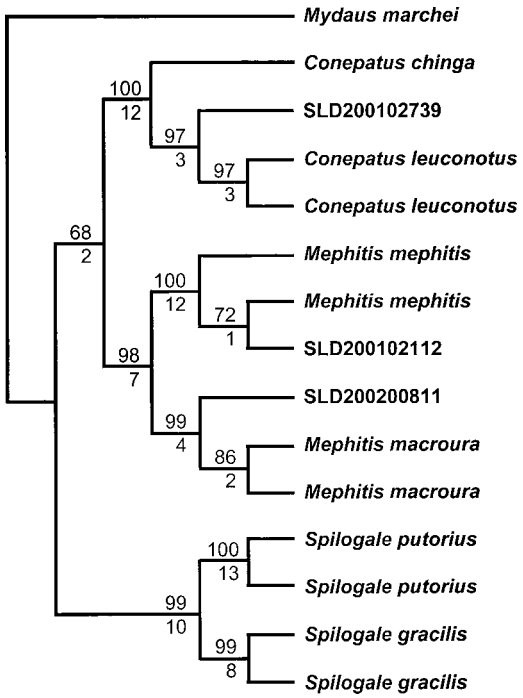

FIGURE 1. An example of the maximum parsimony analyses (heuristic search) obtained from inclusion of three unknown skunk brain samples. Numbers were assigned at the Scientific Laboratory Division, New Mexico Department of Health (Albuquerque, New Mexico). SLD200102739 grouped with white-backed hog-nosed skunk (*Conepatus leuconotus*), SLD200102112 grouped with striped skunk (*Mephitis mephitis*), and SLD200200811 grouped with hooded skunk (*Mephitis macroura*). Numbers above branches represent bootstrap support, whereas numbers below branches represent the number of steps to collapse a node (decay index).

genus/species group of unknown samples is presented as an example of relationships found for each sample (Fig. 1).

Dragoo et al. (1993) and Dragoo and Honeycutt (1997) used multiple mitochondrial genes, including d-loop, as well as nuclear markers to suggest that *Spilogale* and *Mephitis* were sister taxa. Additionally, analysis of the complete d-loop sequence (data not shown) supports those earlier findings. The purpose of this study, however, was not to ascertain relationships among skunk genera but rather to determine if we could identify to species the specimens submitted for rabies testing. The d-loop has enough signal in the first approximately 600 bases to place individuals within one taxon to the exclusion of another.

Most information on prevalence and molecular biology of the rabies virus in terrestrial wildlife comes from animals submitted for testing following human exposure. Little is known about the prevalence of rabies in natural populations and how enzootic or epizootic levels of the disease interact with the ecology of various species (Tinline, 1988; Greenwood et al., 1997).

Much data regarding ecology of skunks and rabies has been collected in Canada and the northern US (Rosatte and Gunson, 1984; Rosatte et al., 1992; Greenwood et al., 1997). These studies only investigated striped skunks or the interactions of striped skunks and raccoons (*Procyon lotor*). However, little is known of the ecology of striped skunks in the arid southwest. Likewise, little is known of the ecology of hooded skunks, hog-nosed skunks, western spotted skunks or the interactions of these four species in Arizona and New Mexico. It is known, however, that skunks maintain an enzootic level of rabies within populations throughout southeastern Arizona, southern New Mexico, and Texas, as well as Mexico, with epizootic outbreaks occurring periodically (Aranda and López-de Buen, 1999).

In order to understand the ecology of rabies, it is imperative that species harboring the virus are correctly identified. Ideally, skins, skeletons, and skulls (voucher specimens) should be collected to properly identify specimens diagnosed with rabies (Ruedas et al., 2000). Significant morphologic differences make it possible to distinguish among North American skunk species (Hall, 1981). Cranial characteristics can be used to distinguish striped and hooded skunks, although they are more difficult to interpret (Hoffmeister, 1986). However, if a voucher specimen is not available, then the next best alternative is to compare DNA from an unknown sam-

ple to DNA from a properly vouchered specimen.

Accurate identification of skunk species will allow for more reliable development of models to understand spread of rabies in these species. For example, Christensen and Bergman (2001) examined a trap-vaccinate-release model when an outbreak of a bat strain of rabies virus occurred in a skunk population in Flagstaff, Arizona. They reported trapping and vaccinating two skunk species, striped and hooded skunks. However, hooded skunks have not been reported previously in that area or habitat. A recent report on the Flagstaff rabies outbreak did not mention hooded skunks (Engeman et al., 2003). Because these animals were released, a voucher specimen could not be obtained. However, blood or hair could have been used as a source of DNA and compared with vouchered specimens to support or refute the existence of hooded skunks in Flagstaff.

Identification of a hog-nosed and a hooded skunk within an extremely small group of samples submitted for rabies testing that were simply labeled "skunk" suggest that skunks can be misidentified at diagnostic laboratories. We have shown that molecular techniques can be used to distinguish species in areas where multiple species are sympatric.

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