

MOLECULAR EVIDENCE FOR GENETIC SUBDIVISIONS IN THE DESERT SHREW, *NOTIOSOREX CRAWFORDI*

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ABSTRACT—Examination of cytochrome-*b* DNA sequences from specimens of *Notiosorex crawfordi*, the desert shrew, indicate that within this taxon there are at least 3 major subdivisions reflecting levels of variation more typical of species rather than subspecies. One of these cytochrome-*b* haplotypes was recently described as a previously unrecognized species; however, that paper was limited to the description of a species level name. We provide additional insight into subdivisions in *N. crawfordi*. One DNA haplotype is distributed in Texas, New Mexico, and eastern Arizona, a second haplotype in Arizona and Sonora, Mexico, and the third haplotype in Baja California, Mexico. Nucleotide sequences obtained from intron 7 of the nuclear gene, beta fibrinogen, support the conclusions that 2 of the haplotypes occur sympatrically in southeastern Arizona and are not hybridizing with each other.

RESUMEN—Examen de las secuencias de ADN del gen citocroma *b* en especímenes de *Notiosorex crawfordi*, la musaraña del desierto, indica que dentro de este taxón se encuentran por lo menos 3 subdivisiones mayores, reflejando niveles de variación típicos de especies y no de subespecies. Uno de los haplotipos del citocroma *b* fue descrito recientemente como una especie no reconocida previamente, pero ese manuscrito se limitó a la descripción de esa nueva especie. Proveemos más información acerca de las subdivisiones dentro de *N. crawfordi*. Un ADN haplotipo habita en Texas, Nuevo México, y el este de Arizona; el segundo habita en Arizona y Sonora, México; y el tercero habita en Baja California, México. Secuencias obtenidas del intron 7 del gen nuclear beta fibrinogéneo, apoyan las conclusiones de que 2 de los haplotipos ocurren simpátricamente en el sureste de Arizona sin evidencia de hibridación.

Members of the *Notiosorex crawfordi* group occur in the southwestern United States and the northern Mexico. The type specimen was collected near Fort Bliss, El Paso Co., Texas, in 1895 (Merriam, 1895). Two subspecies, *N. crawfordi crawfordi* and *N. crawfordi evotis*, were recognized by Armstrong and Jones (1972). Based on morphological data, *N. crawfordi* was divided into 3 species (*N. crawfordi*, *N. evotis*, and *N. villai*) by Carraway and Timm (2000). Recently, Baker et al. (2003) described one of the cytochrome-*b* haplotypes present in *N. crawfordi* (sensu Carraway and Timm, 2000) as *N. cockrumi*. The present study further examined cytochrome-*b* haplotypes and genetic subdivision in shrews defined as *N. crawfordi* by Carraway and Timm (2000).

Baker and Hsu (1970) reported karyotypes from 3 specimens of *N. crawfordi* from Garza County, Texas, and one specimen from Pima

County, Arizona. The specimen from Pima County had a diploid number of 62 and a fundamental number of 94, whereas the specimens from Garza County had a diploid number of 68 and a fundamental number of 102. With limited samples from such widely separated localities, the significance of differences in diploid number and fundamental number were not resolved.

Initially, we sequenced the mitochondrial cytochrome-*b* gene to better understand implications of the chromosomal variation and to examine phylogeographic variation within the *N. crawfordi* group. There is substantial literature documenting utility of the cytochrome-*b* gene for addressing phylogeographic patterns in mammals (Avice et al., 1987; Nazareth et al., 1998; Ohdachi et al., 2001). After examining genetic distance values among haplotypes present in *N. crawfordi* as defined by Carraway and Timm

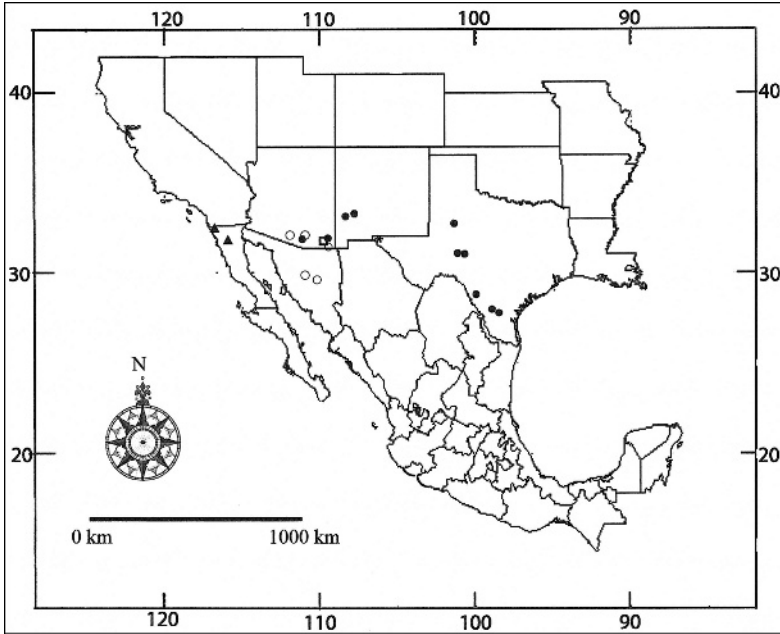


FIG. 1—Distribution of collection localities of *Notiosorex* for which cytochrome-*b* data are available. Open circles identify samples of *N. cockrumi* (clade A) with the open square identifying the type locality. Solid dots identify localities where phylogroups referable to *N. crawfordi* (clade B) were collected. Asterisk identifies the type locality for *N. crawfordi*. Closed triangles identify sites where a third phylogroup (clade C) was collected.

(2000) presented by Bradley and Baker (2001), it was evident that data from a nuclear marker would be valuable in determining whether or not there was breeding between the groups. This especially would be true for populations in southeastern Arizona, where individuals with the 2 haplotypes occur sympatrically. The nuclear gene chosen for this study was intron 7 of the beta-fibrinogen gene (Wickliffe et al., 2003), which has been shown to be phylogenetically informative for several species (Johnson and Clayton, 2000; Weibel and Moore, 2002; Baker et al., 2003; Wickliffe et al., 2003).

**METHODS**—Tissue samples from 30 specimens of *N. crawfordi* were obtained from museum collections of the Natural Science Research Laboratory of the Museum of Texas Tech University, the Museum of Vertebrate Zoology at the University of California Berkeley, and the Angelo State Natural History Collections at Angelo State University (Appendix I). Samples used are from throughout the range of *N. crawfordi* (Fig. 1). Samples used in this study are associated with voucher specimens deposited in mammal collections at their respective museums.

**DNA Extraction and mtDNA Sequencing**—About 0.1–0.5 g of liver tissue were used to extract total genomic DNA by following the procedures described by Long-

mire et al. (1997). Double-stranded amplification of the cytochrome-*b* gene was performed via polymerase chain reaction (PCR). Amplification was accomplished with the following primer combination either L14724 (Kocher et al., 1989) or LGL765 (LGL John Patton) and H15915 (Irwin et al., 1991). Conditions for these reactions were: 95°C for 2 min, 35 cycles of 96°C for 50 s, 50°C for 45 s, and 72°C for 2 min. A final PCR cycle was held at 72°C for 7 min to allow for extension of DNA to occur. The PCR products were purified by use of a Qjagen PCR purification kit (Qjagen, Inc., Valencia, California) following instructions from the manufacturer.

Internal sequencing primers, New3L (5'-CAAAYG-GAGCATCWATA-3'), New4L (5'-CATCCACCCC-TACTACAC-3'), and New5H (5'-GCTAAATGGTCG-GAATA-3'), were designed using Oligo 4.0 (National Biosciences, Inc., Plymouth, Minnesota). Sequencing of the complete cytochrome-*b* gene for the first 14 individuals was performed with terminal primers L14724 (Kocher et al., 1989) or LGL765 (LGL John Patton) and H15915 (Kocher et al., 1989) with the addition of the internal primers. The remaining 16 individuals were sequenced for the proximal 400 base pairs (bp) of the cytochrome-*b* gene via the primers L14724 (Kocher et al., 1989) and H15149 (Irwin et al., 1991). DNA was sequenced in both directions to ensure accuracy of generated sequences by using ABI Big Dye Terminator Ready Reaction Mix (PE Applied Biosystems, Foster City, California), following the instructions of the manufacturer and an ABI Prism

310 Genetic Analyzer (PE Applied Biosystems, Foster City, California).

**Phylogenetic Analyses of Cytochrome *b***—Complete sequences (1,140 bp) of the cytochrome-*b* gene for 14 individuals were examined by alignment of sequence data via Sequencher 3.1 (Gene Codes Corp., Ann Arbor, Michigan). Three types of phylogenetic analyses (maximum-parsimony, maximum-likelihood, and a Bayesian analysis) were performed using either PAUP 4.0b10 (Swofford, 2001) or MRBAYES 2.0 (Huelsenbeck and Ronquist, 2001). Generated trees were rooted using the outgroup criterion with *Sorex cinereus* (GenBank Accession AY128083) as the outgroup. Maximum parsimony analysis was performed with an unweighted character scheme, and 1,000 bootstrap iterations (Felsenstein, 1985) were generated to test node robustness within the tree. Modeltest (Posada and Crandall, 1998) was used to determine the most appropriate model of evolution for our dataset, and parameters were then set to the F 81 (Felsenstein, 1981), with a gamma distribution (F81+G). Bayesian analysis was run with MRBAYES 2.0 (Huelsenbeck and Ronquist, 2001) using the F81+G model of evolution. To evaluate parameters used, an MCMC was run with 4 incremental chains. A starting tree was chosen at random, and  $5.0 \times 10^4$  generations were run with Markov chains sampled every 100 generations with a burn-in of 10 generations producing 4,000 sample points. The resulting trees were used to generate a 50% majority consensus tree with posterior probability values, where values  $>0.95$  were considered significant (Huelsenbeck and Ronquist, 2001).

The remaining 16 individuals then were aligned with the proximal 400 bp from the previously mentioned data and analyzed as above. The resultant trees were used to identify samples from each of the genotypes identified in our study area. Ten of the resulting individuals (TK 49909, TK 49910, TK 49917, TK 49918, TK 49919, TK 49921, TK 49922, TK 49928, TK 84584, and TK 84632) were amplified for a nuclear marker to compare with the cytochrome-*b* data.

**Beta-Fibrinogen Sequence and Data Analysis**—Double-stranded amplification of intron 7 of the beta-fibrinogen gene was obtained via PCR (Wickliffe et al., 2003). Primers used in amplification were  $\beta$ -fib I7L (5'-GTATCTGCCATTAGGGTTGGCTGCATG-3') and  $\beta$ -fib I7U (5'-GACAATTCACAATGGCATGTACTTCAG-3'). Conditions of the PCR were: 93.5°C for 1 min, 33 cycles of 93.5°C for 40 s, 49°C for 40 s, and 72°C for 1.5 min. The final PCR cycle was held at 72°C for 2 min to allow for extension, followed by a 4°C hold. The PCR product was purified using the Qiagen PCR purification kit (Qiagen Inc., Valencia, California) following instructions of the manufacturer. Primers used in the double-stranded amplification subsequently were used in sequencing both strands. Sequences were aligned using Vector NTI Suite 7.1 (Informax Inc., Carlsbad, California) and genetic distance data generated using PAUP 4.0b10 (Swofford, 2001). An individual *S. cinereus* (TK 28042) was amplified and sequenced for intron 7 of the beta-fibrinogen gene and used as an outgroup in all phylogenetic analyses. Maximum-parsimony, maximum-likelihood, and Bayesian analyses were performed as described under cytochrome-*b* data analysis.

**RESULTS—Cytochrome *b***—For the 14 specimens sequenced, the cytochrome-*b* gene was 1,140 bp. Among these specimens, 14 unique haplotypes were present, with corrected divergence values ranging from 0.1 to 14.1%. GenBank accession numbers are AY611562 to AY611591. Within putative species of *N. crawfordi*, of the 1,140 bases compared, 234 (20.5%) were variable, of which 35 (14.9%) were first-position changes, 3 (1.3%) were second-position changes, and 196 (83.7%) were third-position changes.

Results of maximum-parsimony, maximum-likelihood, and Bayesian analysis produced trees with similar topologies with 3 distinct clades. Due to similarities among the 3 trees, one tree (maximum likelihood) was chosen for presentation in this paper (Fig. 2). This tree provided clear evidence of 3 clades with good support for most major nodes (Fig. 2).

Specimens collected from Sonora, Mexico, and southeastern and south-central Arizona comprise clade A (Fig. 2) and represent *N. cockrumi* (Baker et al., 2003). The internal divergence values within this clade ranged from 0.1 to 0.4%, whereas the divergence values between this clade and clades B and C were 12.96 and 13.78%, respectively. Specimens from the largest geographic area, including Texas, New Mexico, and southeastern Arizona are represented by clade B (Fig. 2). Internal divergence values within this clade ranged from 0.0 to 0.1% and divergence values between this clade and the remaining 2 clades, A and C, differed from this clade by values of 12.96 and 13.55%, respectively. Clade C (Fig. 2) is composed of 2 individuals from northern Baja California, Mexico, and has an internal divergence value of 0.1%.

**Beta Fibrinogen**—Sequence data for about 387 bp of intron 7 of the beta-fibrinogen gene for 10 individuals (GenBank Accession AY611592-AY611602) resulted in identification of 2 distinct genotypes. Individuals identified by use of cytochrome-*b* sequence data as belonging to either clade A or clade B are distinguishable from each other by the presence of an 18-bp indel in position 211 to 228, as well as 7 transitions and 3 transversions in the remainder of the sequenced intron. Individuals belonging to clade A (*N. cockrumi*, Fig. 2) do not contain this 18-bp addition, whereas individuals from clade B (*N. crawfordi*) do. Divergence values for the 2 clades were similar, with 1.39% divergence

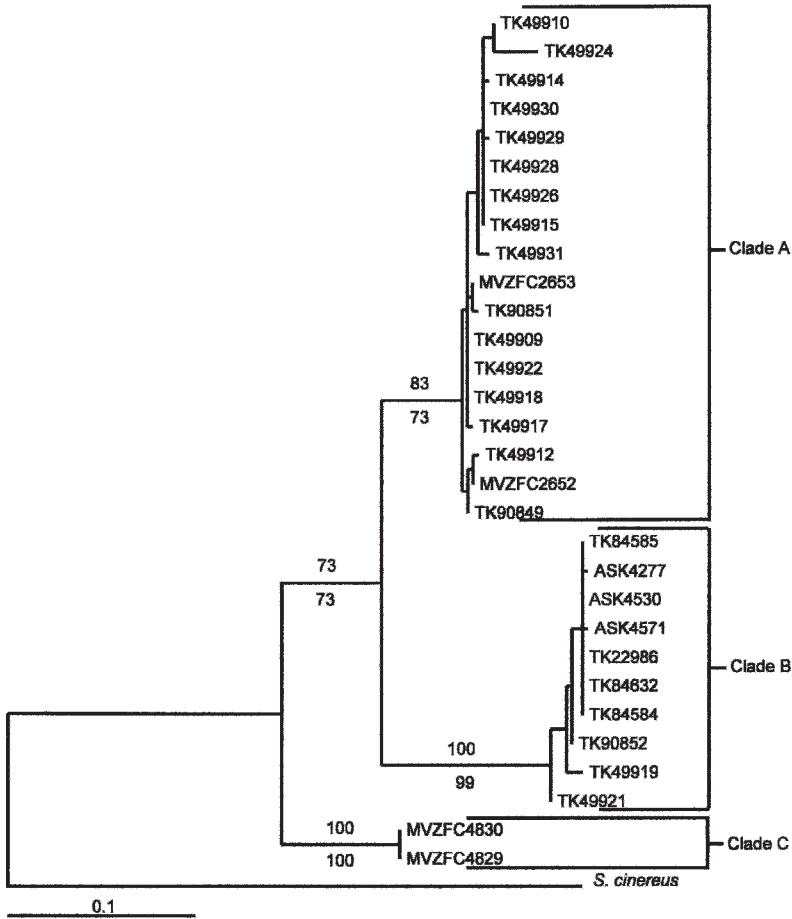


FIG. 2—Maximum-likelihood tree depicting cytochrome-*b* relationships within *Notiosorex crawfordi*, with bootstrap values above the nodes and Bayesian support values below the nodes. Major clades are denoted on the right-hand side of the tree. Clade A represents *N. cockrumi*, clade B represents *N. crawfordi*, and clade C represents a potential new species of *Notiosorex* located in Baja California, Mexico.

among the 6 individuals from clade A and an average divergence value of 1.29% among individuals in clade B. Between clades A and B, diversity values ranged from 2.85 to 6.89%, with an average of 4.40%. With this 18-bp indel, a heterozygote would have been detectable by the presence of a misalignment at position 211 to 228. Such a misalignment was not observed in our sample of 10 individuals.

DISCUSSION—Based on haplotype diversity in the mitochondrial cytochrome-*b* gene, *N. crawfordi* (sensu Carroway and Timm, 2000) consists of at least 3 major genetic subdivisions or phylogroups (Avise and Walker, 1999). The 2 alternative explanations for the variation ob-

served in the cytochrome-*b* gene in *N. crawfordi* are: 1) there is one widespread biological species with polymorphic divergent haplotypes that do not predict variation in the nuclear genome or 2) that the divergent haplotypes are markers of reproductively isolated populations. If reproductively isolated populations are present, then this should be evident in unique alleles within the nuclear genome.

DNA sequences for the 6 samples from clade A (*N. cockrumi*) and 4 samples from clade B (*N. crawfordi*) revealed that most variation in this nuclear marker is partitioned between the 2 haplotypes. Differences were observed in 2 forms: 1) in *N. cockrumi* (clade A) there is an 18-bp fragment missing that is present in *N.*

*crawfordi* (clade B) and 2) when common sequences were aligned for 387 bp, there were differences within the sequences that distinguished the 2 haplotypes. The 2 haplotypes have 4.40% sequence divergence, with 7 transitions and 3 transversions. The values reported here are consistent with those reported by Weibel and Moore (2002) who noted that beta-fibrinogen intron 7 evolved at a rate 4 times slower than the cytochrome-*b* gene and Johnson and Clayton (2000) who reported intraspecific variation among species of Columbiformes ranged from 0.27 to 7.30%.

There are morphological, mitochondrial-DNA sequences from the cytochrome-*b* gene, and nuclear sequences from the beta-fibrinogen gene available for representatives of populations assigned to *N. crawfordi* by Carraway and Timm (2000). What are the implications of each of these datasets, and what are the implications for all datasets when viewed in concert?

**Morphology**—Based on morphology, Carraway and Timm (2000) recognized 3 species; *N. crawfordi*, *N. evotis*, and *N. villai*. Within *N. crawfordi*, they examined 229 specimens from throughout its geographic range. These authors were aware of the genetic differences in the cytochrome-*b* gene, and they examined specimens to determine if morphology reflected these mitochondrial genetic differences. Carraway and Timm (2000:313) stated “Although significant genetic differences occur between populations of *Notiosorex crawfordi* in Baja California and Texas, we found no identifiable morphological differences.” The significance of this observation is that if any morphological difference accompanied the herein described genetic variation, this morphological difference is small and difficult to detect.

**Cytochrome-*b* Gene**—Avisé and Walker (1999) indicated that within most vertebrate species that have been examined for cytochrome-*b* gene variation, there is a major dichotomy in sequence values that are geographically defined (phylogroups). They further concluded that it is probable that most of these divergences indicate the presence of 2 or more biological species. Variation among phylogroups was identified by Johns and Avisé (1998) as being  $>2\%$ , and most of their examples were between 2.5 and 7%. Bradley and Baker (2001) presented additional data on rodents and bats and reported average intraspecific genetic-distance values to be 2.50%,

with a range of 0 to 8.70%. Bradley and Baker (2001) also examined genetic distances between sister species and determined the average was 8.13%, with a range of 2.50 to 19.23%. Within *N. crawfordi*, the distance values ( $>10\%$ ) among clades are at the upper limits presented by Johns and Avisé (1998) and Bradley and Baker (2001). Based on the cytochrome-*b* data alone, *N. crawfordi* has a high probability of comprising 3 biological species. Clade A, *N. cockrumi*, occurs in southern Arizona and central Sonora (Fig. 1). Clade B, *N. crawfordi*, is the most widespread, occurring in Texas, New Mexico, and southeastern Arizona, and Clade C occurs in Baja California, Mexico. The magnitude of differences in the haplotypes observed in *N. crawfordi* was greater than the level of differences distinguishing most sister species among rodents and bats (Bradley and Baker, 2001).

Whereas, among-clade variation in cytochrome-*b* sequence is high ( $>13\%$ ), within-clade variation is relatively small (0.1–0.4%). The implication of this reduced level of intraclade variation is that population bottlenecks or selection has reduced or eliminated mitochondrial variation. Chromosomal differences that distinguish the Arizona specimens from specimens from Texas (Baker and Hsu, 1970) also suggest that there are substantial genetic differences that distinguish members of clade A from clade B. This assumes that the karyotypic differences are associated with the respective clades. This conclusion is probable but remains to be verified.

**Beta-Fibrinogen Intron 7**—Whereas there are substantial data for many species for the cytochrome-*b* gene, there is a much smaller dataset for the beta-fibrinogen gene in mammals. However, these examples suggest that the beta-fibrinogen intron 7 evolves at a rate about one-third that of the cytochrome-*b* gene (Johnson and Clayton, 2000; Weibel and Moore, 2002; Wickliffe et al., 2003). A comparison of distance values for the cytochrome-*b* gene and the beta-fibrinogen gene in *N. crawfordi* are in agreement with those values and, in concert with the cytochrome-*b* data, further indicate a long period of isolation for the 2 phylogroups.

A problem in using maternally inherited mitochondrial data as the only source of identification of morphologically similar species is that polymorphisms might exist in interbreeding populations, which would be undetectable by

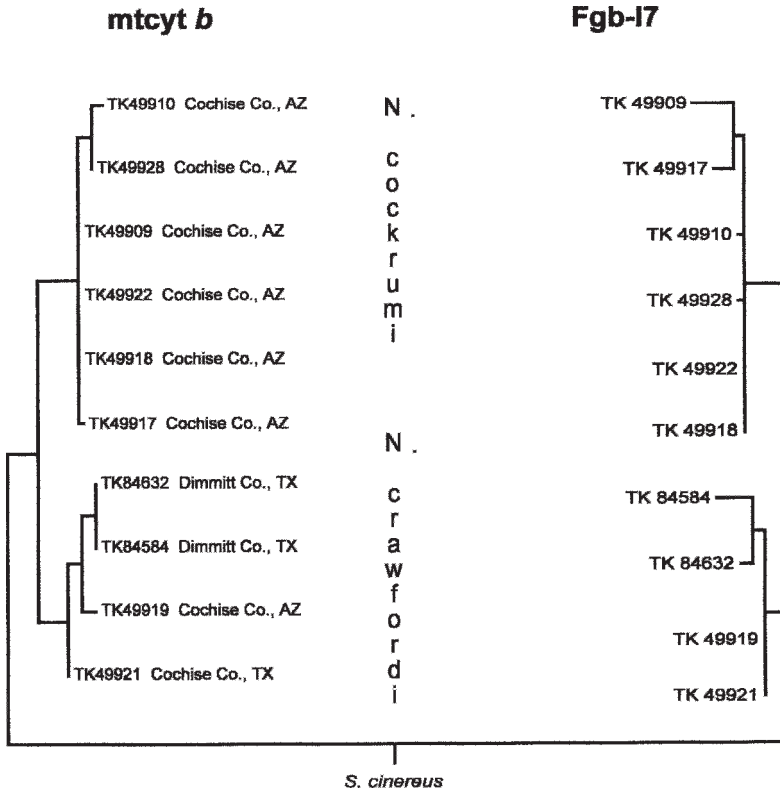


FIG. 3—Comparison of maximum-likelihood trees generated using the model F81+G (Felsenstein, 1981) for *Notiosorex cockrumi* (clade A) and *N. crawfordi* (clade B) samples using the molecular markers cytochrome *b* and beta-fibrinogen intron 7.

a maternally inherited marker. However, if another genetic marker is available that is not linked to the mitochondrial genome, it is possible to detect whether a population is panmictic with polymorphisms or, alternatively, behaving as 2 biological species. For *Notiosorex* from southeastern Arizona, the beta-fibrinogen gene and the cytochrome-*b* gene provide resolution because polymorphisms do not explain the observed variation. Sympatric individuals from southeastern Arizona have associated nuclear and mitochondrial character states, which Baker et al. (2003) interpreted as documenting that the different genetic combinations are behaving as biological species (Fig. 3).

In conclusion, *N. crawfordi* (sensu Carraway and Timm, 2000) is an example where there is little justification for major subdivisions in morphology, but strong evidence in both the mitochondrial and nuclear genome that there are major genetic subdivisions. Based on genetic distance values, the beta-fibrinogen data and the

cytochrome-*b* data are most compatible with the hypothesis that populations of *Notiosorex* in southeastern Arizona represent biological species and that a level of reproductive isolation is in place. Further, the observation that individuals in clades A and B occur in sympatry in south-central Arizona and are maintaining their genetic distinctiveness is further evidence that there are multiple species within *N. crawfordi*. As such, we affirm previous work by Baker et al. (2003) and recommend recognition of 3 species in the current range of *N. crawfordi*. Those individuals representing clade A should be referred to as *N. cockrumi* (Baker et al., 2003), clade B should maintain their designation as *N. crawfordi*, and clade C represents a third, currently unnamed species in need of further study.

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APPENDIX I—Specimens examined and their geographical localities are given below: TK numbers correspond to samples from the frozen-tissue collection at the Natural Science Research Laboratory, Texas Tech University, Lubbock, Texas; MVZFC numbers correspond to samples from the Museum of Vertebrate Zoology, Berkeley, California; ASK numbers correspond to samples from Angelo State Natural History

Collection, San Angelo, Texas. Where available, voucher-specimen numbers precede tissue numbers. Numbers with an asterisk (\*) refer to samples for which we have mitochondrial and nuclear data.

*Notiosorex crawfordi*—MEXICO: 10 m SE El Rosario, Baja California, Mexico (MVZFC 4830); 9 m S Rosarito, Baja California Sur, Mexico (MVZFC 4829); UNITED STATES: 4.5 m W San Angelo, Tom Green Co., Texas (ASK 4530); 5 m N, 9.8 m W Mertzon, Irion Co., Texas (ASK 4571); Devils River State Natural Area, 29°58.4'N, 100°58.2'W, Val Verde Co., Texas (ASK 4277); Leslie Canyon National Wildlife Reserve, T21S, R28E, Section 20, NE¼, Cochise Co., Arizona (TTU 82991\*, TK 49919\*; TTU 82993\*, TK 49921\*); Chaparral Wildlife Management Area, Dimmit Co., Texas (TTU 80965\*, TK 84584\*; TTU 80966, TK 84585; TTU 80807\*, TK 84632\*); Jack Lotts Ranch Post, Garza Co., Texas (TTU 40000, TK 22986); SE Tucson, Exit 275 on I-10, Houghton Road, 7 m S, Pima Co., Arizona (TK 90852).

*Notiosorex cockrumi*—MEXICO: 14.6 m E Mazocahui, Sonora, Mexico (MVZFC 2652); 4.1 m NW Nacari Chico, Sonora, Mexico (MVZFC 2653); UNITED STATES: Leslie Canyon National Wildlife Reserve, T21S, R28E, Section 20, NE¼, Cochise Co., Arizona (TTU 82981\*, TK 49909\*; TTU 82982\*, TK 49910\*; TTU 82984, TK 49912; TTU 82986, TK 49914; TTU 82987, TK 49915; TTU 82989\*, TK 49917\*; TTU 100000\*, TK 49918\*; TTU 82994\*, TK 49922\*; TTU 82996, TK 49924; TTU 82998, TK 49926; TTU 83502\*, TK 49928\*; TTU 83503, TK 49929; TTU 83504, TK 49930; TTU 83505, TK 49931); 1 m past 1012 Southern Pacific Railroad near Cienega Creek, T16S, R17E, NE¼, Pima Co., Arizona (TK 90849); Exit 275 on I-10 or Houghton Road 7 m S Tucson, Pima Co., Arizona (TK 90851).

*Sorex cinereus*—UNITED STATES: 2 m E Cimarron, US Highway 50, Montrose Co., Colorado (TTU 41918\*, TK 28042\*).