A comparison of phylogenetic systematics among Middle American shrews of the genus *Cryptotis* (Mammalia: Soricidae) based on morphological versus molecular data

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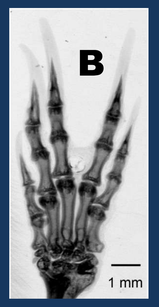
Introduction

Members of the genus *Cryptotis* (Mammalia: Soricidae), the small-eared shrews, are found from eastern North America to Andean South America. The *Cryptotis mexicanus* group is a clade within the genus whose species are distinguished by enlargement of the forefeet and longer, broader foreclaws (Woodman and Morgan 2005). These modifications are believed to confer a distinct digging advantage to *C. mexicanus* group shrews. Forefoot morphology in *C. mexicanus* shrews is thought to be a shared characteristic that groups these organisms together. However, evolutionary relationships among these cryptic species are difficult to define using morphological data. Variation in DNA sequences from the mitochondrial cytochrome b (cyt*b*) gene and the nuclear apolipoprotein (ApoB) gene can be used to elucidate relationships between species and construct a molecular phylogeny (Ohdachi et al 2006; Dubey et al 2008). We sequenced 1140 bp of the cyt*b* gene in 32 individuals and 517 bp of the ApoB gene in 33 individuals across seven *Cryptotis* species. From these data, we generated two maximum parsimony phylogram trees: one based on the cyt*b* data alone, and the other based on 17 concatenated cyt*b* and ApoB sequences. Phylogenies based on these molecular data will help to resolve the relationships of different *Cryptotis* species and confirm the morphological species delimitation.

Figure 1A. Specimens of *C. mam* from field work in 2008.

Figure 1B. X-ray photograph of the left manus of *C. mam*. X-ray photographs were used to develop a morphological phylogeny for *Cryptotis*.



Materials and Methods

Cryptotis tissue samples originating from localities in Guatemala, Costa Rica, Colombia, and the United States were obtained from fresh tissue samples and from voucher specimens in the USNM collection (Table 1). Genomic DNA was extracted from the tissue samples and the 1140 bp cytochrome b gene was amplified using the LGL 765 and LGL 766 primers in a polymerase chain reaction (PCR). I used two sets of internal primers (L14724 and H15149; L15162 and H15915) to amplify a 400 bp and a 700 bp fragment respectively for samples that failed to amplify the entire cyt*b* with primers LGL 765 and LGL 766. In addition, I used ApoB primers (ApoBF and ApoBR) specifically developed for shrews (Dubey et al 2008) to amplify 517 bp of the ApoB gene. PCR products were cycle-sequenced and purified. Both strands of each sample were sequenced using the ABI PRISM 3130 Genetic Analyzer. Sequencher 4.9 was used to align and edit sequences.

Thirty-two sequences were successfully obtained for the cyt*b* gene, and 33 sequences were successfully obtained for the ApoB gene. Twelve additional cyt*b* sequences and four additional ApoB sequences from GenBank were used to supplement the analysis. Partial sequences (N=1) and single stranded sequences (N=3) were used when the entire cyt*b* sequence could not be produced. PAUP* 4.0 was used to perform phylogenetic analyses. Duplicate haplotypes were omitted from further analysis. A preliminary neighbor-joining (NJ) tree was generated based on all cyt*b* sequences. A maximum parsimony (MP) analysis with 1000 bootstrap replicates and maximum likelihood (ML) tree were generated from the cyt*b* data, with *Blarina brevicauda*, *B. carolinensis*, and *B. hylophaga* included as the closest outgroup species as per Ohdachi et al 2006; Dubey et al 2008. Seventeen representative cyt*b* sequences from the original data set were concatenated with the ApoB sequences and an MP heuristic analysis and MP bootstrap analysis with 100,000 replicates of these data were performed.

Results

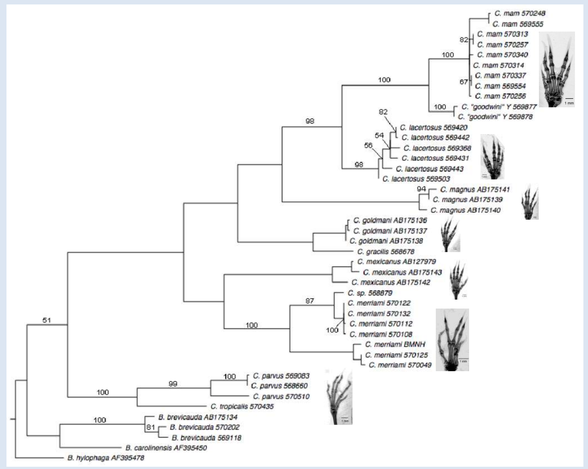


Figure 2. Maximum parsimony heuristic phylogram of 39 *Cryptotis* cytochrome *b* sequences, with five *Blarina* outgroup sequences. Bootstrap supporting values are indicated by the numbers near nodes. X-ray photographs of *Cryptotis* forefoot morphology are included adjacent to clades.

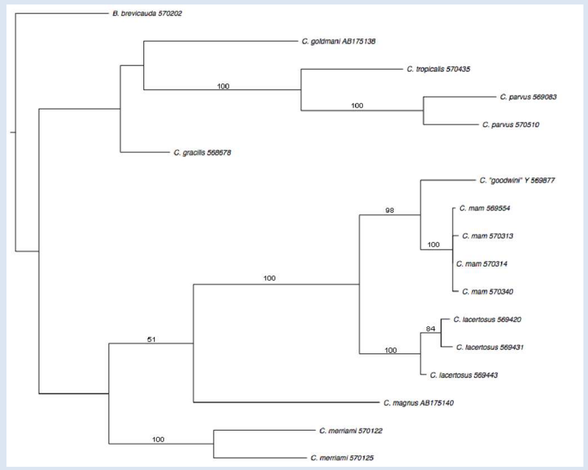


Figure 3. Maximum parsimony heuristic phylogram of 17 concatenated cyt*b* and ApoB sequences, with *B. brevicauda* as the outgroup. Samples for this analysis were chosen based on the availability of sequences of both genes and even representation of clades formed in Figure 1. Numbers near nodes indicate bootstrap support. Table 1 highlights sequences analyzed in this phylogram in yellow.

Table 1. List of all museum specimen sequences and GenBank sequences used in the cyt*b*-based phylogeny with respective localities. Sequences highlighted in yellow were included in the concatenated cyt*b*/ApoB MP phylogeny (Figure 2).

Species	Specimen Number	GenBank Accession Number	Locality
C. mam	570248	--	Huehuetenango, Guatemala
	570340	--	Huehuetenango, Guatemala
	570337	--	Huehuetenango, Guatemala
	569554	--	Huehuetenango, Guatemala
	570314	--	Huehuetenango, Guatemala
	570256	--	Huehuetenango, Guatemala
	570313	--	Huehuetenango, Guatemala
	570257	--	Huehuetenango, Guatemala
	569555	--	Huehuetenango, Guatemala
C. "goodwini" Y	569877	--	Alta Verapaz, Guatemala
	569878	--	Alta Verapaz, Guatemala
C. lacertosus	569420	--	Huehuetenango, Guatemala
	569442	--	Huehuetenango, Guatemala
	569368	--	Huehuetenango, Guatemala
	569431	--	Huehuetenango, Guatemala
	569443	--	Huehuetenango, Guatemala
	569503	--	Huehuetenango, Guatemala
C. magnus	--	AB175141	Oaxaca, Mexico
	--	AB175139	Oaxaca, Mexico
	--	AB175140	Oaxaca, Mexico
C. goldmani	--	AB175136	Guerrero, Mexico
	--	AB175137	Guerrero, Mexico
	--	AB175138	Guerrero, Mexico
C. gracilis	568678	--	Cartago, Costa Rica
C. mexicanus	--	AB127979	Oaxaca, Mexico
	--	AB175143	Oaxaca, Mexico
	--	AB175142	Oaxaca, Mexico
C. sp.	568879	--	Valle del Cauca, Colombia
C. merriami	570122	--	Alta Verapaz, Guatemala
	570132	--	Baja Verapaz, Guatemala
	570112	--	Alta Verapaz, Guatemala
	570108	--	Alta Verapaz, Guatemala
	BMNH	--	Alta Verapaz, Guatemala
	570125	--	Zacapa, Guatemala
	570049	--	Zacapa, Guatemala
C. parvus	569083	--	Virginia, USA
	568660	--	Virginia, USA
	570510	--	Kansas, USA
C. tropicalis	570435	--	Huehuetenango, Guatemala
B. brevicauda	--	AB175134	Michigan, USA
	570202	--	Maine, USA
	569118	--	Virginia, USA
B. carolinensis	AF395450	--	Georgia, USA
B. hylophaga	AF395478	--	Nebraska, USA

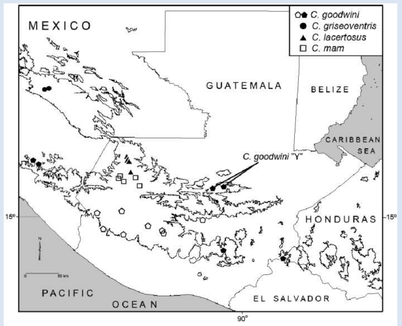


Figure 4. Map indicating the distributions of *C. goodwini* (examined species shown by open pentagons, literature records by closed pentagons), *C. goodwini* "Y" (indicated on the map), *C. lacertosus* (triangles), *C. mam* (squares), and *C. griseoventris*, another species in the *C. mexicanus* group. The 1500-m contour is shown. Adapted from Woodman, 2010.

Discussion

Heuristic MP analyses of both the cyt*b* and concatenated cyt*b*/ApoB datasets revealed a highly supported monophyly of the three *C. goodwini*-like species from Huehuetenango and Alta Verapaz, Guatemala—*C. lacertosus*, *C. mam*, and *C. goodwini* "Y". The relative positions of these species in the cyt*b* molecular phylogeny concord with a recently published morphological phylogeny, which places them as the members of the *C. goldmani* subset of the *C. mexicanus* group (Woodman 2010). However, these nodes are not strongly supported by the bootstrap analysis.

The molecular position of *C. goldmani* presents a larger problem. Enlarged forefeet and modifications in the humerus suggest that *C. goldmani* is a highly derived member of the *C. mexicanus* group. A molecular analysis of cyt*b* in four species of *Cryptotis* supported this relationship (Ohdachi et al 2006). In this study, both phylogenies place *C. goldmani* in a more primitive position than expected from morphological data. The phylogeny based on cyt*b* data places *C. goldmani* in a clade with *C. gracilis* and ancestral to *C. magnus* and the derived members of the *C. goodwini* subset. The cyt*b*/ApoB analysis places *C. goldmani* in a position basal to even *C. parvus*, the least morphologicaly derived species of *Cryptotis* in this study (Woodman and Morgan 2005). However, because these basal nodes do not have strong bootstrap support, additional nuclear intron data are necessary to confirm the relationships of these groups.

Two distinct clades of *C. merriami* emerge from this analysis—one comprising four specimens from Alta Verapaz and Baja Verapaz, and the other comprising two specimens from Zacapa and one from a unique locality in Alta Verapaz. These clades were also highly supported by the bootstrap analysis.

The molecular data also revealed anomalies in morphological classification. One specimen (*C. sp.*, 568879) unexpectedly grouped with a geographically and morphologically distinct species (*C. merriami*) and is awaiting further morphological evaluation.

Our molecular analysis of shrew phylogeny clarified relationships within species, and confirmed morphological species delimitation among the *C. goodwini*-like species (Woodman 2010). Other relationships were not resolved through mitochondrial and nuclear data and resulted in a polytomy. In addition, we found that several samples shared identical haplotypes across regions. For these reasons, continued sampling of *Cryptotis* species in Central America and sequences from more nuclear markers are necessary to resolve the phylogenetics of Middle American *Cryptotis*.

Acknowledgments

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