Original Study

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Geometric morphometrics clarifies the taxonomic status of semifossorial shrews (Eulipotyphla, Soricidae, Cryptotis) from Mexican cloud forests

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Abstract: The small-eared shrews Cryptotis mexicanus and Cryptotis obscurus (Eulipotyphla, Soricidae) are two closely related taxa from the northern Neotropics whose taxonomy is still unresolved. Here, we tested the hypothesis of three lineages (Northern, Central, and Southern) within this pair of semifossorial shrews. We photographed skulls, dentaries, and humeri from 226 museum specimens and used geometric morphometrics to evaluate sexual dimorphism, differences between lineages, and the effect of the environment on the shape of the structures. We found negligible differences between males and females, supporting previous studies of classical morphometrics in this genus. Our results also support the differentiation of semifossorial shrews into the three geographically isolated groups, where the size of the dentary and the shape of the three examined structures contribute to discrimination. We found a significant sign of the influence of the environment on the shape of the dentary but not on the skull or the humerus. We suggest that geographic isolation across climatically similar regions has contributed to the differentiation between lineages. We propose recognizing all three lineages as valid species that must be adequately described.

Keywords: allometry; allopatric speciation; climate; Cryptotis; Neotropics

1 Introduction

Shrews (Eulipotyphla, Soricidae) are the fourth most diverse family of mammals in the world (Burgin et al. 2018), behind murid and cricetid rodents (Muridae and Cricetidae) and vespertilionid bats (Vespertilionidae). In tropical and subtropical regions, taxonomic studies on shrews are still in progress, so the number of species discovered and described has increased notably in recent years (Camargo and Álvarez-Castañeda 2020; Esselstyn et al. 2021; Guevara 2023), and there are still rearrangements and new taxonomic proposals within some diverse genera.

In the Neotropics, small-eared shrews (genus Cryptotis) is the most diverse and widely distributed group, especially in wet and cold regions from central Mexico to Guatemala (Woodman and Timm 2017). In Mexico, a natural grouping of species has received the name of the Cryptotis mexicanus group, until recently composed of Cryptotis nelsoni, Cryptotis magnus, C. mexicanus, Cryptotis obscurus, and Cryptotis phillipsii (Guevara and Cervantes 2014). As in other shrew species of the Blarinini tribe, this group is characterized by its semifossorial habits (Woodman and Wilken 2019). It is highly associated with the humid montane forest (or cloud forest) along the Sierra Madre Oriental (SMO), the Trans-Mexican Volcanic Belt (TVB), Sierra Madre del Sur (SMS), and the Chiapas Highlands (Guevara et al. 2015). The most common and widely distributed species are C. mexicanus and C. obscurus, two closely related taxa whose taxonomy has been controversial and is still unresolved (Choate 1970; Guevara and Cervantes 2022; Woodman and Timm 1999).

Recently, the synonymy of C. obscurus with C. mexicanus was suggested due to their high molecular, morphological, and ecological similarity (Guevara and Cervantes 2014; Guevara and Sánchez-Cordero 2018). Despite this, we must note that there is an evident differentiation in skull size
across different populations (see Figure 4 in Guevara and Sánchez-Cordero 2018). A recent phylogeographic study with the Cytochrome b mitochondrial gene revealed the possible recognition of three lineages throughout the distribution of this group of shrews that inhabit similar environmental conditions but are isolated by barriers: (Vázquez-Ponce et al. 2021): (1) Northern, from the northernmost population in Tamaulipas to the eastern zone of the FVT; (2) Central, from the center of the SMO and the east region of the FVT to the Santo Domingo river, Oaxaca; and (3) Southern, from the south of the Santo Domingo River in the SMS to the Chiapas Highlands, east of the Isthmus of Tehuantepec (Figure 1). Therefore, it is necessary to collect more data and evaluate them with other taxonomic approaches to better elucidate the taxonomic and geographic status of this component of the *C. mexicanus* species group.

A complementary approach to address taxonomic problems in morphologically complex taxa is the study of shape through geometric morphometrics (Calahorra-Oliart et al. 2021; Dashti et al. 2022; Ospina-Garcés and León-Paniagua 2022), which is based on multivariate statistical tests where the spatial relationship among different structures of organisms is evaluated (Adams et al. 2004). Geometric morphometrics has been used in some taxonomic studies within the three subfamilies of shrews (Jacquet et al. 2013; Matthews and Stynder 2011; Shchipanov et al. 2016; Zidarova and Popov 2018). A common aspect in these studies has been using the skull, mandible, and some dental pieces to assess the differences between populations and species (Tse and Calede 2021; Zidarova and Popov 2018), showing that this method can be helpful in the assessment of taxonomic problems.

In this research, we use geometric morphometrics for evaluating morphological differentiation and test the proposal of three lineages within *C. mexicanus* and *C. obscurus* (Northern, Central, and Southern; sensu Vázquez-Ponce et al. 2021). In addition, we analyze the relationship between environmental factors and the change in the shape of bone structures. With the above, we contribute to clarifying the taxonomic status and better understand the evolution of these shrews associated with humid mountain ecosystems.

2 Materials and methods

2.1 Data collection

We examined 226 specimens of *C. mexicanus* and *C. obscurus* housed in four mammal collections (Supplementary Table S1): Colección Nacional de Mamíferos, Instituto de Biología, Universidad Nacional Autónoma de México, México City (CNMA); The University of Kansas Natural History Museum, Lawrence (KU); Museo de Zoología “Alfonso L. Herrera,” Facultad de Ciencias, Universidad Nacional Autónoma de México, México City (MZFC); and the National Museum of Natural History, Smithsonian Institution, Washington (USNM). Of the 226 specimens, 42 correspond to the Northern, 69 to the Central, and 115 to the Southern lineage. The examined specimens were assigned to each group based on geographic location and recognition of barriers (see above).

We photographed the ventral view of the skull, lateral view of the left dentary, and ventral view of the left humerus using a Canon LS1 camera and a 50 mm lens for the skull and dentary and 22 mm for the humerus. We obtained 411 photographs, of which 181 correspond to the skull, 106 to the dentary, and 124 to the humerus. For each specimen, we recorded the sex and the collecting locality, to later be grouped into the three lineages. Due to the impossibility of photographing the specimens from Los Altos de Chiapas (part of the southern lineage), we did not include them in the present study. Cranial, dental, and postcranial terminology follows Woodman and Timm (1999) and Reed (1951).

2.2 Morphometric data

We used two-dimensional scanning for all three structures. For the skull, we used a configuration of 20 landmarks (Figure 2a), and for the dentary, 11 landmarks (Figure 2b). In both cases, the landmarks were located in the region corresponding to the dentition due to their importance in feeding (Ungar 2015). For the humerus, a structure highly associated with the semifossorial locomotion of *Cryptotis* shrews, we used 15 landmarks and 49 semilandmarks coordinates in seven
curvatures (Figure 2c). Descriptions of the landmarks are provided in Supplementary Table S2.

The digitization of landmark configurations on images of the total sample was carried out with the program tpsDig Version 2.16 (Rohlf 2015). In the contours of the humerus, semilandmarks were located equidistantly using the drawing curve tool in tpsDig, and the semilandmark alignment protocol was made in tpsUtil Version 1.81.

To eliminate the shape variance due to scale, location, and rotation, and to obtain aligned coordinates derived from landmark configurations, we performed a Generalized Analysis of Procrustes, in which the configurations of all the individuals are rotated, positioned on the same axis, and readjusted to the same scale to make them comparable. The semilandmarks of the humerus were aligned using the bending energy criterion (Webster and Sheets 2010). We also obtained the centroid size (CS) as a size estimator of each geometric configuration, which is calculated as the squared sum of the distances of each landmark to the centroid of the configuration (Zelditch et al. 2012). Aligned configurations and CS were used to analyze shape and size variation in relation to geographic lineages and environmental data.

2.3 Statistical analysis

To evaluate the effect of the different variables and factors on the shape configurations of the views, we ran a Procrustes ANOVA model. To assess how much shape variance is explained by CS differences, we carried out a model in which the response variable was the shape, and the explanatory variable was the CS; then, we tested sexual dimorphism, eliminating from the analysis the specimens whose sex could not be determined. For CS, we performed an ordinary least-squared model to determine if there was a size difference among the previously proposed three groups. Subsequently, all these tests were performed considering the sum of squares and cross products of type I (sequential) and 999 iterations, using the package geomorph version 4.0.4 (Baken et al. 2021).

Additionally, to evaluate the size differences in the structures along the lineages, we also carried out a linear model in which we used the CS as a response variable and the origin of the specimens as an explanatory variable. This was using the RRRP package version 1.1.2, considering 999 iterations (Collyer and Adams 2019), and making a boxplot to visualize the differences detected.

To determine if there were differences among the three lineages, we carried out a model of the shape given the a priori grouping. We first performed a Principal Component Analysis (PCA) for each structure to reduce the number of explanatory variables, preserving the greatest variation of the shape (Supplementary Figures S1–S3). Then, we performed a Canonical Variable Analysis (CVA) based on the first ten principal components of shape variance for each geometric configuration to test group differences. To estimate the power of discrimination of geographic groups, we calculated the percentage of reassignment of specimens to their original group from linear discriminant analysis (Curran 2012) through the cross-validation parameter. Additionally, we obtained pairwise Mahalanobis distances between groups and tested their significance using a permutation procedure of one thousand replicates on the original groups using the package MASS (Venables and Ripley 2013).

Finally, a previous study showed a broad overlap of climatic niches between C. mexicanus and C. obscurus (Guevara and Sánchez-Cordero 2018). However, it is unknown if the climate could exert any pressure on the morphological variation along the distribution of the lineages. Here, to determine if there is a relationship between climate and shape variation of different structures among lineages, we performed a partial least squares (PLS), establishing the degree of covariance between variables (Ospina-Garcés and León-Paniagua 2022; Rohlf and Corti 2000); therein we used 19 climatic variables that describe the average and extreme conditions of precipitation and temperature as covariates of the shape of each specimen associated with each of the lineages in the three bone structures. The variables were used at a resolution of 1 km² and were downloaded from the WorldClim web portal (Fick and Hijmans 2017).

3 Results

Tests for sexual dimorphism did not show differences between males and females in any of the three structures (skull, p > 0.05, r² = 0.017; dentary, p > 0.5, r² = 0.027; humerus, p > 0.5, r² = 0.027). In the case of the comparison between females and males by geographical area, there were no significant differences either (Table 1). Therefore, all subsequent analyzes were performed without considering the sex.

Significant differences were found in shape given by the size in all the structures, but the values of those differences indicate that the allometric component is very low (skull, p < 0.01, r² = 0.016; dentary, p < 0.01, r² = 0.015; humerus, p > 0.01, r² = 0.014). However, when analyzing the size differences between the lineages, the dentaries of the specimens of the Central lineage turned out to be smaller than those of the Southern and Northern ones (Table 1). On the other hand, the Northern lineage had the most significant differentiation in size, including the largest specimens. The Southern lineage had an intermediate size and the smallest variation among individuals (Figure 3).
In all the structures examined, it was found that there are significant differences between the three lineages (skull, p < 0.01, r^2 = 0.156; dentary, p < 0.01, r^2 = 0.115; humerus, p < 0.01, r^2 = 0.08). In the skull, there were differences between all groups, although the most remarkable difference was found between the Northern and Southern lineages. The regions where the greatest variation was observed are along the molars and the foramen magnum (Figure 4). The allocation percentages obtained in the CVA were 57.44% for the Central, 64.51% for the Northern, and 90.29% for the Southern lineages. The population that presented the greatest variation in the first canonical variable was the Southern, while it was the Central in the second canonical variable (Table 2).

In the dentary and humerus, differences were also found among all groups, with the most remarkable difference observed between the Northern and Southern lineages. The differences in the dentary are located mainly in the region of the coronoid process and the molars. In this case, the highest allocation percentage was for the Southern lineage (76.32%), followed by the Central (72.5%) and the Northern (67.86%; Figure 5). For both the skull and the dentary, the representation of the canonical variables shows the overlap between all lineages; however, the first axis of variation helps to discriminate the Northern and Southern lineages better.

The variation for the humerus was primarily located in the region of greater and lesser tuberosity and the epi-condyle (Figure 6). The humerus obtained the highest average allocation percentage compared to the other two structures (93.67%, Southern; 74.07%, Central; 72.22%, Northern). As for the canonical variables, the first had more significant variation in the specimens of the Southern group, while the second variable had greater dispersion in the Northern. It can be observed in the analysis of canonical variables that the first canonical axis differentiates mainly the Central from the Southern lineage. In contrast, the differences are between the Central and Northern lineages. In summary, the analysis of the three structures indicates that the three lineages can be distinguished by their shape.

The PLS regression with precipitation and temperature variables indicated that the skull and humerus did not show significant covariance (skull, p > 0.05, r^2 = 0.27; humerus,

Table 1: Results of the Procrustes ANOVA testing for sexual dimorphism, allometry, and size among the three lineages (Northern, Central, and Southern) of the shrews Cryptotis mexicanus (squares) and C. obscurus from Mexico.

<table>
<thead>
<tr>
<th>Shape – sex</th>
<th>Pr(&gt;F)</th>
<th>r^2</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skull</td>
<td>0.139</td>
<td>0.018</td>
<td>1.238</td>
</tr>
<tr>
<td>Dentary</td>
<td>0.523</td>
<td>0.027</td>
<td>0.946</td>
</tr>
<tr>
<td>Humerus</td>
<td>0.228</td>
<td>0.028</td>
<td>1.16</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Shape – centroid size</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skull</td>
<td>0.003</td>
</tr>
<tr>
<td>Dentary</td>
<td>0.002</td>
</tr>
<tr>
<td>Humerus</td>
<td>0.028</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Centroid size – lineage</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skull</td>
<td>0.728</td>
</tr>
<tr>
<td>Dentary</td>
<td>0.001</td>
</tr>
<tr>
<td>Humerus</td>
<td>0.514</td>
</tr>
</tbody>
</table>

Figure 3: Box plots showing variation in dentary size among the three lineages (Northern, Central, and Southern) of semifossorial shrews of Mexico. The thick line represents the mean of the data.
p > 0.5, r^2 = 0.14). In contrast, there was significance in the dentary with a covariance percentage of 43% (p < 0.005, r^2 = 0.436). The Central lineage had the least dispersion on the climatic axis (X-axis). On the other hand, the Southern lineage was the one that had the greatest dispersion, as well as a tendency to have the lowest values. As for the shape (Y-axis), the Northern lineage is the one that presented a lower variation in the dentary (Figure 7).

### Table 2: Mahalanobis distances obtained at the linear discriminant analyses for each structure.

<table>
<thead>
<tr>
<th>Lineage comparison</th>
<th>Mahalanobis distances</th>
<th>Reclassification discriminant function (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skull</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northern—Central</td>
<td>1.77</td>
<td>65–77</td>
</tr>
<tr>
<td>Northern—Southern</td>
<td>2.98</td>
<td>65–90</td>
</tr>
<tr>
<td>Central—Southern</td>
<td>2.27</td>
<td>57–90</td>
</tr>
<tr>
<td>Dentary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northern—Central</td>
<td>1.92</td>
<td>68–73</td>
</tr>
<tr>
<td>Northern—Southern</td>
<td>2.69</td>
<td>68–76</td>
</tr>
<tr>
<td>Central—Southern</td>
<td>1.98</td>
<td>73–76</td>
</tr>
<tr>
<td>Humerus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northern—Central</td>
<td>2.5</td>
<td>72–74</td>
</tr>
<tr>
<td>Northern—Southern</td>
<td>2.5</td>
<td>72–94</td>
</tr>
<tr>
<td>Central—Southern</td>
<td>2.86</td>
<td>74–94</td>
</tr>
</tbody>
</table>

Percentage obtained in the reclassification discriminant function for each orno- of the lineages of the shrews Cryptotis mexicanus and C. obscurus from Mexico. P-values equal or less than 0.01 in all cases.

### 4 Discussion and conclusions

#### 4.1 Differences between females and males

The results indicate that the differences in size and shape between males and females are insignificant in the three structures evaluated (Table 1), which is consistent with previous studies based on classical or geometric morphometrics. For example, practically no differences between sexes were found in species of Crocidura and Neomys, two genera distantly related to Cryptotis (Zidarova 2015). In the common shrew, Sorex araneus, a species widely distributed in Europe and Asia, the differences in size are subtle and only for some adult jaw variables (Mishta and Searle 2019; Nováková and Vohralík 2017). Previous studies have not found notable differences between sexes in the genus Cryptotis, although it is important to mention that sample sizes have been small (Choate 1970; Woodman and Timm 1993). More recently, a study with C. nelsoni, a species closely related to C. mexicanus, found no discernible differences between males and females (Guevara and Cervantes 2022).

#### 4.2 Evolution of C. mexicanus and C. obscurus

The geometric morphometrics analysis supports the differentiation of at least three lineages within C. mexicanus and C. obscurus, previously proposed based on the phylogeographic analysis of the Cytochrome b (Vázquez-Ponce et al. 2021). Here, the allometry tests for the three structures were insignificant, indicating that if there are differences in size,
these are not correlated with the change in shape. In the skull and humerus, the size of the structures did not allow differentiation between the three lineages proposed by Vázquez-Ponce et al. (2021). In the case of the dentary, there was a significant difference, particularly distinguishing the Central lineage with the smallest average size. This phenomenon has been observed in other groups of shrews of the Sorex and Neomys genera, in which it was possible to discriminate between species based on the size and shape of the jaw (Rychlik et al. 2006; Vasil’ev 2015).

The shape of the three structures makes it possible to recognize the three lineages (Figures 4–6). Differences in the skull, particularly the dentary, could be associated with diet specialization since differences in shape affect bite force (Tse and Calede 2021). It is important to highlight that, in the case of the dentary, not only the shape varies between the three lineages, but also the size. Consequently, the selection and use of prey could vary between lineages. In the case of shrews with semifossorial habits, such as the C. mexicanus group, modifications have been described.
in the humerus that could be related to food-foraging activities and shelter underground (Woodman and Morgan 2005). Differences in the humerus have proven to be helpful in discovering and describing species in the genus Cryptotis (Woodman 2010), as observed in the present study.

From a biogeographic point of view, it has been mentioned that Quaternary glacial-interglacial cycles have promoted changes in the geographic distribution of small-eared shrews with an effect on the morphological and genetic variation among populations (Choate 1970; Woodman and Timm 1999). Fossil records of *C. mexicanus* 100 km further north-northwest of its current range are evidence of distribution shifts during the Late Pleistocene (Arroyo-Cabrales et al. 2021). Some valleys and canyons that cross perpendicularly to the Sierra Madre Oriental and the northern Sierra Madre del Sur could act as barriers in interglacial phases due to their warmer conditions that do not allow the establishment of humid habitats (Guevara and Sánchez-Cordero 2018; Woodman and Timm 1999).

During glacial stages, these barriers could vanish due to the expansion of forests toward lower-elevation areas (Guevara 2020). However, for species of small size and with low dispersal capacity, such as shrews (Schloss et al. 2012), this forest expansion does not guarantee individuals’ movement and the possible gene flow between populations. The prolonged geographic isolation could favor differentiation due to selective processes or genetic drift, allowing for variation in size and shape independently between lineages.

Previous examinations based on the analysis of qualitative characteristics or classical morphometrics failed to recognize the relatively subtle differentiation between the three lineages (Guevara and Sánchez-Cordero et al. 2018; Ramírez-Pulido et al. 2014); therefore, the taxonomic status of both species has been waiting to be resolved. Now, with the contribution of mitochondrial DNA and the shape of the bone structure, it is possible to have greater certainty of the evolution within this group of shrews and resolve their taxonomic status.

The environmental analysis provided evidence to understand the morphological variation throughout the distribution. Differences in the dentary between lineages as a response to climate could have repercussions on the foraging strategies under different climatic conditions (Rychlik et al. 2006). It remains to be explored whether other environmental variables (not assessed here, such as the diet or soil characteristics) could help us understand the morphological adaptation of lineages to the heterogeneous conditions found throughout cloud forests in the northern Neotropics (Ruiz-Jiménez et al. 2012). The Central lineage that inhabits under lower temperatures and a smaller climatic range is expected to be a particularly climate-endangered species under climate change conditions (Foden et al. 2019; Nadeau and Urban 2019).

### 4.3 Taxonomic conclusions

Our geometric morphometrics data support the hypothesis of Vázquez Ponce et al. (2021) that there are at least three lineages within the *C. mexicanus*-*C. obscurus* group. The Northern lineage that covers Tamaulipas to the east of the TVB should be named *C. mexicanus* (s.s.), while *C. obscurus*...
would become a junior synonym. The Central lineage would have a restricted distribution between the Pico de Orizaba and the Santo Domingo River, Oaxaca, and would be pending to be named. Finally, the Southern lineage would be an endemic species to the cloud forests of northern Oaxaca and awaiting a name. The population to the east side of the Isthmus of Tehuantepec, tentatively grouped with the southern lineage (Vázquez-Ponce et al. 2021), was not analyzed here. However, it is possible that it also represents an independent species recognized by qualitative and quantitative characteristics and long-term geographic isolation (Guevara and Sánchez-Cordero 2018), although with low genetic differentiation (Guevara and Cervantes 2022; Vázquez-Ponce et al. 2021). With these proposed taxonomic changes, the genus Cryptotis would become the most diverse genus of shrews in Mexico, with 20 species, followed by the genus Sorex. The formal description of each mentioned lineage is pending, collecting all available information.

Research ethics: No live animals were used in this study. The curators-in-charge granted permission to examine and photograph voucher specimens.

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Conflict of interest statement: The authors declare that they have no conflicts of interest regarding this article.

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