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Exploring species boundaries of the spotted ground squirrel (*Xerospermophilus spilosoma*) complex

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Xerospermophilus spilosoma exhibits notable geographic and morphological variation, prompting a debate over its taxonomic status. Currently, it is unclear whether it represents a single highly variable species or a complex of cryptic species that includes *X. perotensis*. Although the latter has a larger body size and a distinctive dorsal pattern, current genetic analyses do not support its recognition as a separate species. This study aimed to delimit potential evolutionary units within the *X. spilosoma* complex. Twenty-four sequences of the mitochondrial *cytochrome b* gene were analyzed using 690-bp fragments from *X. spilosoma* and *X. perotensis* specimens collected from eight locations. Phylogenetic and divergence time inferences were estimated using Maximum Likelihood and Bayesian Inference, along with analyses of genetic distances and haplotype networks. Three species delimitation methods (ABGD, PTP, and GMYC) were applied, and the ecological uniqueness and areas of overlap within and between species of the *X. spilosoma* complex were assessed. Four lineages comprising 22 unique haplotypes were identified, with interpopulation genetic distances ranging from 3 % to 6 %. Species delimitation methods suggested between one and four potential species. Meanwhile, the comparison of ecological niches revealed limited overlap. Genetic and environmental evidence indicate that *X. spilosoma* comprises at least three evolutionarily independent lineages. The complex originated in the Miocene, more than 5 million years ago, with divergence events concentrated between 3.5 and 1.5 million years ago, in accordance with geographical barriers such as Río Grande and the Nazas River and the Trans-Mexican Volcanic Belt. These results highlight the need to conserve these populations as independent evolutionary units, particularly the Perote population, given its isolation and ecological and genetic uniqueness.

Keywords: Chihuahuan Desert; *cytochrome b*; Río Grande; Taxonomy.

Xerospermophilus spilosoma presenta una notable variación geográfica y morfológica, lo que ha generado debate sobre su estatus taxonómico. Actualmente se discute si representa una única especie o un complejo de especies crípticas que incluiría a *X. perotensis*. Aunque esta última presenta mayor tamaño corporal y un patrón dorsal distintivo, los análisis genéticos actuales no respaldan su reconocimiento como especie válida. El objetivo de este trabajo fue delimitar las posibles unidades evolutivas dentro del complejo *X. spilosoma*. Se analizaron 24 secuencias del gen mitocondrial *citocromo b,* usando fragmentos de 690 pb de *X. spilosoma* y *X. perotensis,* de ocho localidades distintas. Las inferencias filogenéticas y de tiempo de divergencia se estimaron mediante Máxima Verosimilitud e Inferencia Bayesiana, junto con como análisis de distancias genéticas y redes de haplotipos. Se aplicaron tres métodos de delimitación de especies (ABGD, PTP y GMYC), además de evaluarse el nicho ecológico por especie y sobreposicionamiento entre especies del complejo *X. spilosoma*. Se identificaron cuatro linajes con 22 haplotipos únicos con distancias genéticas interpoblacionales entre el 3 y el 6%. Los métodos de delimitación de especies sugieren entre una y cuatro especies potenciales. Por su parte, la comparación de los nichos ecológicos mostró un bajo solapamiento de áreas. La evidencia genética y ambiental obtenida sugiere que *X. spilosoma* corresponde a un complejo de al menos tres linajes evolutivamente independientes. El complejo se originó en el Mioceno, hace más de 5 Ma, con eventos de divergencia concentrados entre 3.5 y 1.5 Ma, en concordancia con barreras geográficas como los ríos Grande y Nazas y la Faja Volcánica Transmexicana. Estos resultados, resaltan la necesidad de conservar estas poblaciones como unidades evolutivas independientes, especialmente la de Perote, por su aislamiento y singularidad ecológica y genética.

Palabras clave: *Citocromo b*; Desierto Chihuahuense; Río Grande; Taxonomía.

Establishing the boundaries between species is a difficult task, especially in taxa for which speciation processes have not resulted in an evident morphological differentiation (Goldstein and De Salle 2011; Fišer *et al.* 2018). This can be explained by phenomena such as convergence (Losos 2008, 2011), stabilizing selection (Gould 2002; Hansen and Houle 2004), or a recent speciation process (Gittenberger 1991; Rundell and Price 2009). A major problem in taxonomy is that species boundaries vary widely depending on the species concept employed (De Queiroz 2007). Therefore, a unified concept based on their evolutionary origin has been proposed, defining them as evolutionarily independent lineages. These lineages are identified by evaluating secondary characteristics, e.g., ecological, morphological, and genetic traits, that reflect some degree of evolutionary independence (Simpson 1961; Wiley 1978; Bock 2004; Hey 2006; De Queiroz 2007).

With more than 2600 species, the order Rodentia is the most diverse group of mammals worldwide (D'Elía *et al.* 2019). This diversity is partly due to their high evolutionary rates and rapid radiation processes, often associated with evolutionary convergences, which has produced taxonomic complexities that still require resolution (Triant and De Woody 2006; Fabre *et al.* 2012; Burgin *et al.* 2018). Within this order, squirrels of the family Sciuridae stand out for their diversity, with about 300 species distributed in a wide range of ecosystems, from deserts to tropical forests, and from sea level to more than 4500 meters above sea level. This family exhibits great morphological variation, including arboreal, terrestrial, and gliding forms, with solitary or social patterns (Koprowski *et al.* 2016; Rocha *et al.* 2016). In addition, their biological characteristics, such as low dispersal capacity, short life cycles, and varied reproductive strategies, allow for a detailed analysis of the differentiation and evolutionary isolation processes, making squirrels ideal models for genetic and evolutionary studies (Rocha *et al.* 2016; Flores-Manzanero and Vázquez-Domínguez 2019; Waterman *et al.* 2021).

The current distribution of most squirrel species in North America is explained by allopatric speciation processes, where geographic barriers such as mountain systems and water bodies have limited gene flow (Harrison *et al.* 2003; Ge *et al.* 2014; Zelditch *et al.* 2015). For example, the Snake River, in the northwestern United States, has influenced the diversification of small-eared ground squirrel species of the genus *Urocitellus* (McLean *et al.* 2025). In this context, several current species have been isolated in valleys due to geological events that occurred during the Quaternary period, such as fluctuating glaciations or volcanic activity (Harrison *et al.* 2003; Van Tuinen *et al.* 2008; Menéndez *et al.* 2021). A peculiar case is that of the Mojave ground squirrel, *Xerospermophilus mohavensis*, whose possible origin involved allopatric speciation in a small isolated refuge within the Mojave Desert, delimited by the Sierra Nevada (Bell *et al.* 2010).

However, geographic isolation does not always imply sufficient ecological or genetic divergence to justify the separation into distinct species (Barton 2020; Kulmuni *et al.* 2020). An example is the population of Mearns squirrels *(Tamiasciurus mearnsi)* in Baja California, for which genetic studies indicate a relatively recent separation and a limited ecological differentiation despite being geographically isolated from *Tamiasciurus douglasii*, leading to the question of whether *T. mearnsi* should really be considered a distinct species (Arbogast *et al.* 2001; Pecnerová and Martinova 2012; Hope *et al.* 2016). Therefore, examining the niches of species in the environmental space and projecting them in the geographical space constitutes an additional line of evidence to evaluate whether populations, in addition to being physically separated, have occupied and exploited different environments, which could indicate processes of ecological differentiation that support or facilitate the delimitation of species (Peterson *et al.* 2000; Kozak and Wiens 2006; Pahad *et al.* 2019). Similarly, niche modeling studies contribute to understanding the relationship between genetic variation patterns and the environmental parameters that limit the distribution of a species (Ashrafzadeh *et al.* 2018; Calixto-Pérez *et al.* 2018; Luna-Aranguré and Vázquez-Domínguez 2020).

A taxonomic review of the genus *Spermophilus* based on morphology and sequences of the *cytochrome b* (*cytb*) mitochondrial gene revealed its paraphyletic condition, which led to the reassignment of several species to new genera. Thus, *Xerospermophilus* was recognized as a genus that includes *X. mohavensis*, *X. tereticaudus*, *X. spilosoma,* and *X. perotensis* (Helgen *et al.* 2009). However, the phylogenetic relationship between *X. spilosoma* and *X. perotensis* remains a matter of debate, particularly with regard to their recognition as separate species (Harrison *et al.* 2003; Helgen *et al.* 2009; Fernández 2012).

Xerospermophilus spilosoma is the most divergent species that is distributed over a wide geographic range, from Wyoming, South Dakota, and Nebraska to central Mexico (Lacher *et al.* 2016; Álvarez-Castañeda 2024). This wide distribution has favored a high morphological diversity within the species, including variation in coat coloration from cinnamon to dark smoky-gray shades, through various shades of brown. In addition, it has a series of spots on the back and flanks whose shape and intensity vary depending on the environment, probably in response to local ecological conditions (Cothran *et al.* 1977; Álvarez-Castañeda 2024). As a result of this morphological variation, 13 nominal subspecies have been described (Helgen *et al.* 2009; Mammal Diversity Database 2025).

The taxonomic classification of *X. spilosoma* is currently a subject of debate. It is not clear whether it is a single species with high population variability that includes several subspecies (Helgen *et al.* 2009; Fernández 2012), including *X. perotensis*, or if the possible paraphilia observed between the subspecies of *X. spilosoma* and *X. perotensis* indicates that it could be a complex of species (Harrison *et al.* 2003).

Although *X. perotensis* has a larger body size, differences in coloration with a distinctive pattern of dorsal spots and distinct vocalizations, the genetic analyses available to date, based on nuclear (GHR and IRBP) and mitochondrial (Cytb and 12S rRNA) genes, suggest that genetic divergence would not be sufficient to support its recognition as a separate species (Fernández 2012). However, it is worth noting that these conclusions were based on an limited number of specimens, namely 3 individuals of *X. spilosoma* and 4 of *X. perotensis*. Therefore, the objective of this study was to evaluate the differentiation of populations within the *X. spilosoma* complex and their possible status as species using four distinct lines of evidence: (1) phylogenetically delineating the evolutionary units; (2) estimating genetic distances between populations to quantify their divergence; (3) applying species delimitation methods that allow the identification of separate evolutionary lineages; and (4) comparing the degree of ecological differentiation between populations.

Materials and methods

Sample collection. A total of 23 tissue samples were processed, from specimens collected in the field (n = 12) and from specimens obtained from different national and international scientific collections (n = 11). The field collection consisted of ectomization of the third phalanx from *X. perotensis* and *X. spilosoma* specimens captured in Perote and Mapimí, respectively. Sample collection followed the recommendations of Romero-Almaraz *et al.* (2007) and the guidelines of the Committee on Animal Care and Use of the American Society of Mammalogists (Sikes *et al.* 2016). The corresponding collection permits were issued by the Board of the Environment and Natural Resources (SPARN/DGVS/04074/23 and SPARN/DGVS/08359/23). The tissues were fixed separately in 96% ethanol and stored at -70 °C until processing.

Genomic DNA (gDNA) was extracted from the tissues using the DNeasy Blood and Tissue Kit (QIAGEN, USA) according to the manufacturer's instructions. Subsequently, the concentration of the genetic material was quantified in a Nanodrop spectrophotometer (model DS-11, Denovix). To perform the amplification of *cytb* fragments by Polymerase Chain Reaction (PCR), a new pair of oligonucleotides was designed in PerlPrimer v1.1.21: Xeros_Fw (5'YSAYTTACMYGCACCYTCC-3') and Xeros_Rv (5'GGRTATWCAACRGGTTGYCMTC 3'), which amplifies a 981-bp fragment. This primer pair was evaluated *in silico* using SnapGene v.8.1 to verify its specificity, hybridization efficiency, and absence of secondary structures.

The PCR reactions were run in a final volume of 25 µL per sample, containing 2 µL of gDNA (with concentrations between 20 and 200 ng/µL), 14.25 µL of nuclease-free $H_{2O,}$ 6.25 µL of Master mix DreamTaq (QIAGEN, USA), and 2 µL of each primer at 10 µM. One positive control and one negative control were included in each run to ensure procedural reliability and to rule out contamination. The amplification protocol consisted of an initial denaturation step at 94 °C for 5 minutes, followed by 40 denaturation cycles at 94 °C for 30 seconds, alignment at 56 °C for 30 seconds, extension at 72 °C for 70 seconds, and a final elongation step at 72 °C for four minutes. The PCR tests were performed on a BioRad T100 thermal cycler. PCR products were visualized on 2 % agarose gels, stained with red gel, and examined under UV light using a transilluminator. The PCR products were then purified using the Qiaquick Gel Extraction Kit (QIAGEN, USA) according to the manufacturer's instructions. The purified products were sent for bidirectional sequencing to Macrogen, South Korea.

In total, 24 *Xerospermophilus* samples were considered for subsequent analyses. Of these, 12 were generated in this study (7 for *X. spilosoma* and 5 for *X. perotensis*), and 12 were recovered from GenBank. The sequences were edited manually, then aligned using the MUSCLE algorithm, and visually inspected using Geneious Prime and MEGA X (Kumar *et al.* 2018). The final alignment included 14 sequences for *X. spilosoma*, 10 for *X. perotensis* (Appendix 1), and one for *Ictidomys mexicanus* as an outgroup (Harrison *et al.* 2003; Guevara-Chumacero *et al.* 2006; Fernández 2012).

Phylogenetic analyses and genetic diversity. From the global alignment, the best substitution model was selected through an exhaustive search in JModelTest v2.1.10 (Darriba *et al.* 2012). Phylogenetic inference was performed under the Maximum Likelihood (ML) criterion using the previously selected model (GTR + G), with estimated branch support across 10 000 ultrafast bootstrap replicates (Hoang *et al.* 2018) in IQ-TREE v1.6.12 (Nguyen *et al.* 2015). Bayesian Inference (BI) was performed in MrBayes v.3.2.7 (Ronquist *et al.* 2012) with the MCMC algorithm and the previously calculated substitution model (GTR + G + I); to this end, two separate runs were performed with three hot chains and one cold chain with 10 million generations and a 25 % burn-in. Chain convergence and good sampling (ESS > 200) were assessed in TRACER v1.7 (Rambaut *et al.* 2018). The consensus trees generated in each inference were visualized and edited using FigTree v1.4.4. (Rambaut 2010).

Subsequently, a haplotype file was generated in DnaSP v6 (Rozas *et al.* 2017), from which the number of haplotypes was identified, and haplotype diversity (Hd) and nucleotide diversity (Pi) were calculated. With this information, a haplotype network was constructed using the TCS criterion in PopArt v1.7 (Leigh *et al.* 2015). Additionally, an analysis of intra- and interclade genetic distances was performed using uncorrected pairwise sequence distances (p-distances) using the *ape* library (Paradis *et al.* 2004). Based on these data, a heat map was drawn up with the *adegenet* and *split* libraries (Jombart 2008; Ezard *et al.* 2009), both in R v4.4.1 (R Core Team 2020). Genetic distances within the genus *Xerospermophilus* were evaluated using 15 sequences of *X. tereticaudus* and 10 sequences of *X. mohavensis* (Harrison *et al.* 2003; Bell *et al.* 2010; Fernández 2012).

Divergence times. With the alignment constructed as described above, divergence times were inferred in BEAST v2.6 (Bouckaert *et al.* 2019), considering a non-correlated lognormal relaxed molecular clock and replacing *I. mexicanus* for *Cynomys ludovicianus* (Appendix 1) as the outgroup (Castellanos-Morales *et al.* 2014). GTR substitution models with the gamma distribution and the Bayesian Coalescent Skyline plot model were established.

A secondary calibration based on four nodes was performed. The first node was calibrated based on the fossil record of *Cynomys rafinesque*, with a maximum age of 1.8 Ma (Ge *et al.* 2019), representing the divergence between *Cynomys* and the *X. spilosoma* complex. The second node corresponds to the separation between the Kansas, Texas, and New Mexico populations, in the USA, and those of Arizona and Mexico, attributed to the formation of the Rio Grande, whose average consolidation is estimated to have occurred 2.6 Ma ago (Morgan and Golombek 1984; Repasch 2017). The third node reflects the divergence between the populations of Arizona, USA, and Mapimí, Mexico, in contrast to the populations of the Central Plateau of Mexico, associated with the Nazas River, whose origin dates back to the Pliocene and with its current configuration estimated to have occurred 1.5 Ma ago (Petersen 1976; Hafner and Riddle 2011). Finally, the fourth node represents the isolation of the Perote population, in Mexico, attributable to the culmination of the formation of the Trans-Mexican Volcanic Belt, completed approximately 1.6 Ma ago (Ferrari *et al.* 1999, Ferrari 2000; Gámez *et al.* 2017).

All nodes were assigned a lognormal distribution as a prior, as this model reflects the observation that evolutionary divergence typically precedes both the first fossil record and the complete establishment of a geographic event. Under this perspective, speciation does not occur at the same time that a fossil appears or a barrier is formed; instead, these elements represent only minimum age limits (Ho 2007). MCMC analyses were performed with two independent runs of 10 million generations each, sampling every 1000 generations. The convergence, stability, and adequate sampling of the results were evaluated with Tracer v1.7.1 (Rambaut *et al.* 2018), applying a 25 % burn-in. The final phylogenetic tree that included the divergence time intervals was generated using TreeAnnotator v2.6.2 (Bouckaert *et al.* 2019). The graphs were generated using the *deeptime* (Gearty 2025) and *ggplot2* (Wickham 2016) packages in R.

Species delimitation. Boundaries between species within the *X. spilosoma* complex were evaluated using three delimitation methods: the Automatic Barcode Gap Discovery (ABGD), which is based on the identification of a barcode corresponding to the natural discontinuity between intraspecific (minor) and interspecific (major) genetic variability. ABGD groups sequences according to this discontinuity, without the need for an *a priori* hypothesis about the number of species (Puillandre *et al.* 2012). The Poisson Tree Processes (PTP) method, based on a maximum likelihood model, assesses differences in substitution rates between clades to detect clusters that are consistent with putative species (Zhang *et al.* 2013), while the Generalized Mixed Yule Coalescent (GMYC) method uses an ultrametric tree to analyze branch lengths and determine whether they correspond to intraspecific or interspecific processes (Fujisawa and Barraclough 2013).

For ABGD, the previously calculated genetic distance matrix was used. The configuration of the parameters was (i) a minimum intraspecific distance (Pmin) of 0.001 and a maximum intraspecific distance (Pmax) ranging from 0.02 to 0.1; (ii) a Barcode Gap Width of 1.5; and (iii) the Jukes-Cantor model (JC69); for the analysis, we used the command line in the ABGD program (Puillandre *et al.* 2012). For PTP, we used the ML tree generated with 100 000 MCMC generations and a 10 % burn-in on the PTP web server (http://species.h-its.org/). For GMYC, we used an ultrametric guide tree of the genus *Xerospermophilus* generated in MEGA X (Kumar *et al.* 2018). A multiple method was applied using a λ = 5 to fit the molecular-clock model and evaluated confidence intervals (0 - 2); the model was performed using the *splits* package in R (Ezard *et al.* 2009).

Niche modeling. In addition to the genetically analyzed samples, we included 404 records of the presence of *X. spilosoma* and *X. perotensis* from GBIF (2024). These records were organized according to the identification of terrestrial ecoregions proposed by Olson *et al.* (2001) corresponding to each clade defined in the phylogenetic analyses and the delimitation of potential geographical barriers between records: the Rio Grande between clades A and B, the Nazas River between clades B and C, and a minimum convex polygon for clade D, subsequently intersected with the aforementioned ecoregions. A 6 km buffer was established for each polygon delimited by these ecoregions and barriers. This distance was set given the low mobility reported for these squirrels, whose mean dispersal distance is approximately 1.5 km (maximum 2.8 km) (Montero-Bagatella and González-Romero 2014). For clades A and D, the delimitation was obtained directly with this procedure. For clade B, a northern limit was established considering a 30 km buffer with respect to the clade A polygon and applying a 21 km reduction to the south. For clade C, a 21 km reduction to the north was applied, maintaining the previous delimitation to the south, which delineated a 30 km strip between the clades A, B, and C polygons. Based on these polygons, records were classified and grouped for modeling by clades, excluding those located within the 30 km strips or outside the polygons; this procedure was carried out in ArcMap v.10.5.

The ecological niche models were constructed using the 19 bioclimatic variables available in the WorldClim platform (WorldClim 2024), which are in raster format with a 30 arcsec (1 km^2) resolution. The models were generated using the Maximum Entropy algorithm, implemented in MAXENT v3.4.1 (Phillips *et al.* 2017). First, a general model was constructed for each clade to identify the most representative variables. To this end, we used the contribution and permutation percentage table, as well as the Jackknife test, both available in the MAXENT outputs, to assess the relative importance of the explanatory variables. Based on the variables selected by clade, a consensus was performed, selecting those relevant to the four clades.

Subsequently, a Pearson correlation analysis was performed on the previously selected variables. Highly correlated variables (r > 0.85) were excluded to avoid collinearity and reduce redundant information (Segurado *et al.* 2006). We selected seven correlated variables with a simpler environmental interpretation that do not combine humidity and temperature data: BIO1 (mean annual temperature, °C), BIO2 (mean diurnal temperature range), BIO4 (temperature seasonality, %), BIO10 (mean temperature of the warmest quarter, °C), BIO11 (mean temperature of the coldest quarter, °C), BIO15 (seasonality

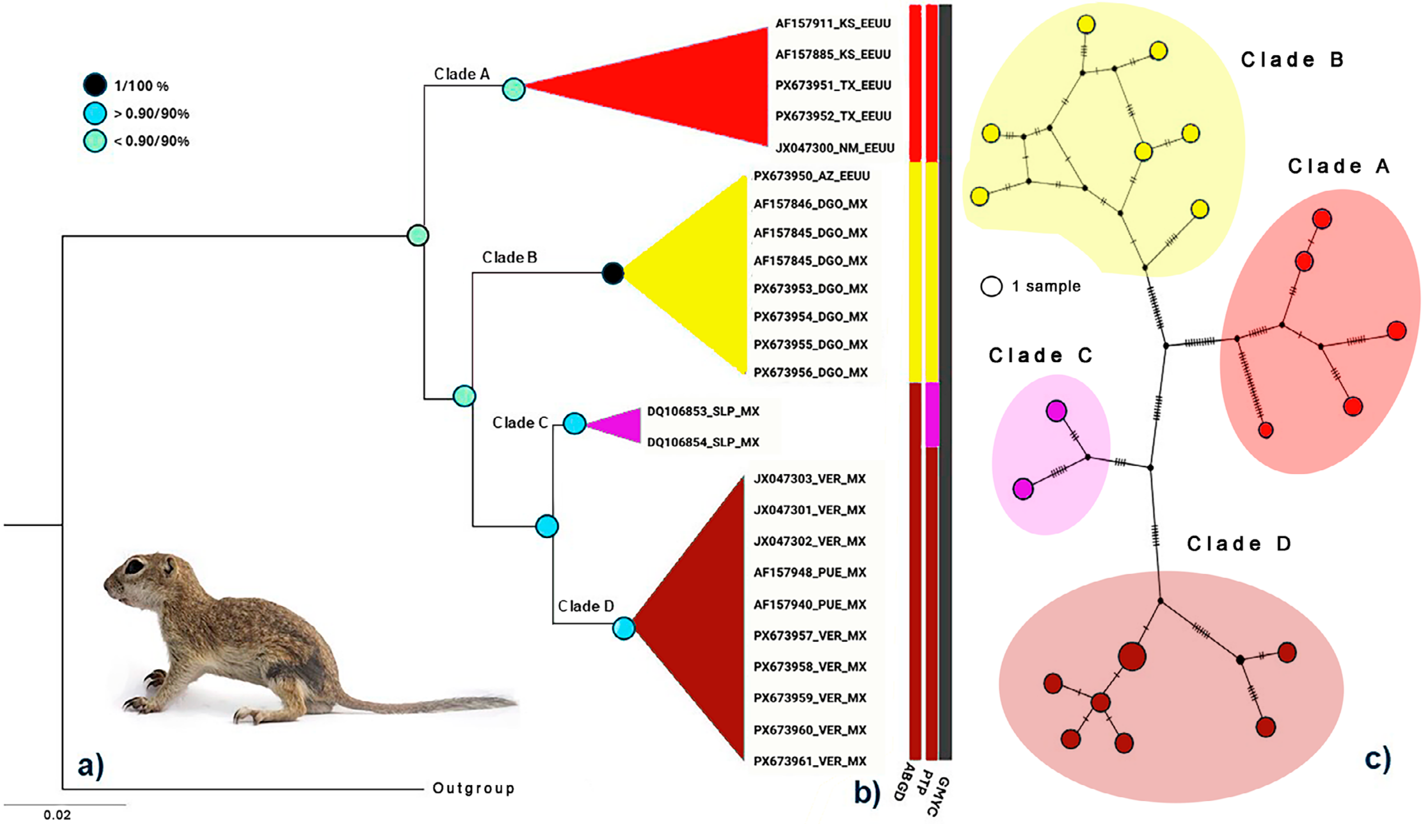


Figure 1. Inferred phylogenetic tree for the *Xerospermophilus spilosoma* complex based on mitochondrial *cytochrome b* gene sequences (690 bp) using the BI and ML methods (a). Circles represent post-bootstrap probability values. (b) Results of species delineation analyses suggesting the presence of between 1 and 4 potential species within the *X. spilosoma complex.* (c) Haplotype networks observed for the different populations analyzed; lines perpendicular to the main branches indicate the estimated number of evolutionary steps.

of precipitation, variability index), and BIO16 (precipitation of the wettest quarter, mm).

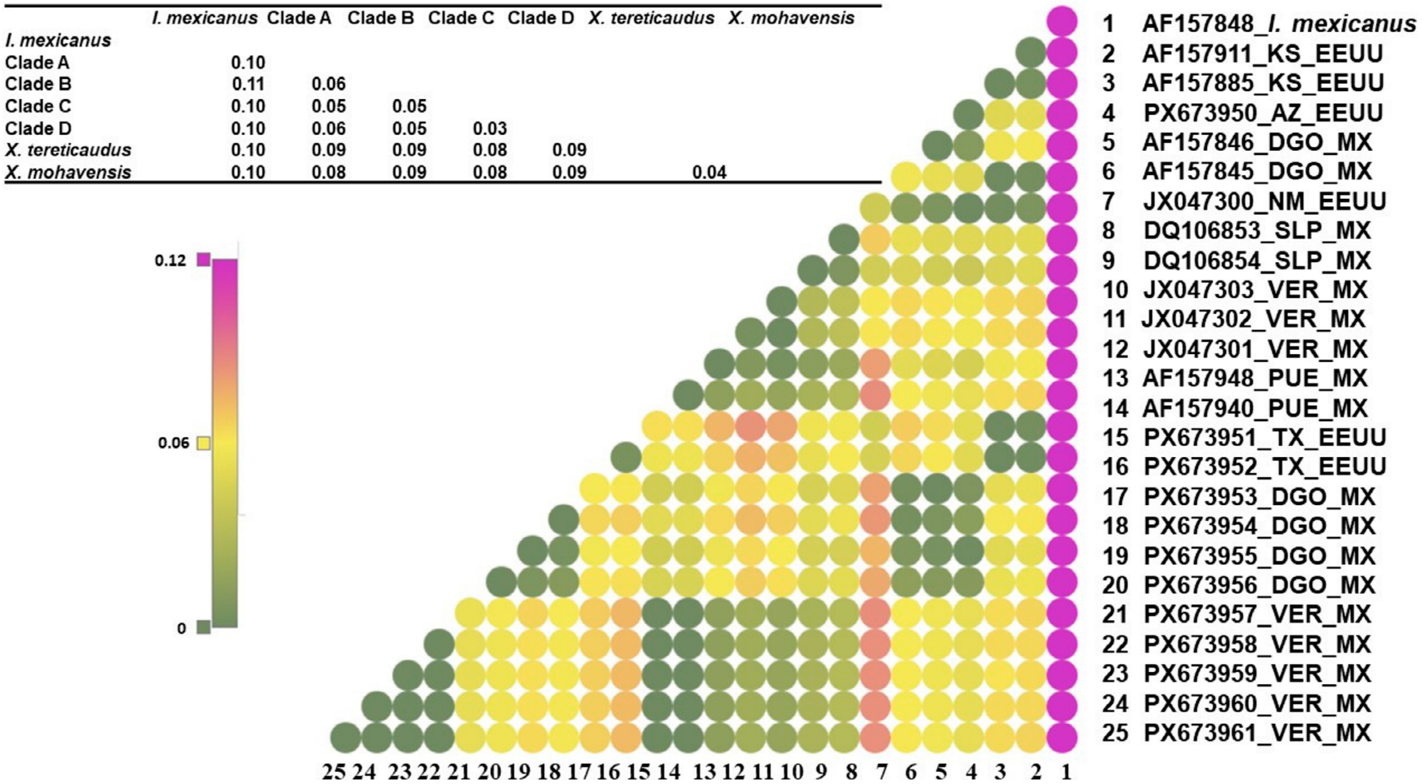
Once the clade-based groups were defined, we performed a 1 km spatial filtering of the final records (clade A, 151; clade B, 72; clade C, 37; clade D, 37) using the *Wallace* package (Kass *et al.* 2018). Then, the models for each group were calibrated using the accessibility or mobility area with 20 000 randomly selected background pixels. This selection represents a hypothesis about the area to which the species has or has had access to disperse (Barve *et al.* 2011). Subsequently, a spatial partitioning of occurrence data was performed to train and validate the models. The method implemented was the chessboard, considering an aggregation factor of 2 (Muscarella *et al.* 2014). Models with linear, quadratic, and combined functions were fitted using regulation multipliers between 0.5 and 3 (0.5 intervals), selecting the best models according to the AIC (Burnham and Anderson 2002). From these, binary (presence/absence) distribution maps were generated according to the biogeographic provinces of Olson *et al.* (2001).

Additionally, niches were characterized in a multivariate space using a Principal Component Analysis (PCA), performing the ordination through a correlation matrix and using Mahalanobis distance approximations (Broennimann *et al.* 2012). The degree of overlap or divergence between the ecological niches of the candidate species was evaluated using similarity and equivalence statistical tests implemented in the *ecospat* v4.2.1 package (Di Cola *et al.* 2017). In both cases, the overlap between niches was quantified using Schoener's-*D* index, whose values range from 0 (completely discordant niches) to 1 (identical niches) (Schoener 1970; Warren *et al.* 2008).

The niche similarity analysis assesses whether the niches of two species are more similar than expected by chance, accounting for the environmental context in which they occur. In this test, a *P*-value < 0.05 suggests that niches are more similar than expected by chance (Broenniman *et al.* 2012). In contrast, in the niche equivalence test, the observed *D*-value is compared to a null distribution constructed from random reassignments of occurrences of each species pair (Brown and Carnaval 2019). In this test, a *P*-value < 0.05 indicates that the niches are more different than expected by chance. For each test, 100 replicates were performed to generate a null distribution of overlap scores, which was compared to the observed values.

Results

The final alignment comprised 690 bp, with 146 variable sites, 81 of which were parsimony-informative. The ML and BI topologies were consistent, recovering the same phylogenetic relationships and with support values greater than 0.85 / 85 %, respectively. Four clades were identified (Figure 1a): clade A includes sequences from the USA populations (Kansas, Texas, and New Mexico); clade B, from Arizona, USA, and Durango, Mexico; clade C only includes sequences from San Luis Potosí, Mexico; finally, clade D



	I. mexicanus	Clade A	Clade B	Clade C	Clade D	*X. tereticaudus*	*X. mohavensis*
I. mexicanus							
Clade A	0.10						
Clade B	0.11	0.06					
Clade C	0.10	0.05	0.05				
Clade D	0.10	0.06	0.05	0.03			
X. tereticaudus	0.10	0.09	0.09	0.08	0.09		
X. mohavensis	0.10	0.08	0.09	0.08	0.09	0.04	

Figure 2. Heatmap showing genetic distances (p) for the *Xerospermophilus spilosoma* complex. Interspecific genetic distances >0.10 are marked in pink, and intraspecific distances ≤0.6 are marked in green, according to the scale. The table presents the genetic distances (p) between the clades and the species close to *X. spilosoma*.

includes sequences from the Perote valley in Puebla and Veracruz, Mexico.

A total of 22 unique haplotypes were recovered, with a haplotype diversity (Hd) of 0.989 and a nucleotide diversity (Pi) of 0.04360. The haplotype network showed four clusters corresponding to the clades observed in the phylogenetic relationships (Figure 1c). These clusters were separated by different numbers of mutational steps (7-14), indicating clear genetic differences between them. Likewise, a high number of mutational steps were observed between the Arizona and Mapimí populations, as well as between the New Mexico and Texas–Kansas populations. In addition, several hypothetical haplotypes were identified that connect the various clusters. On the other hand, the genetic distances between *I. mexicanus* and the remaining sequences analyzed were low (10–11 %*)*. In *X. spilosoma*, intrapopulation genetic variability ranged from 1 % to 3 %, whereas interpopulation variability ranged from 3 % to 6 % (Figure 2).

The estimated divergence times suggest that the *X. spilosoma* complex originated toward the end of the Miocene, just over 5 Ma ago (Figure 3). Most divergence events are concentrated between 3.5 and 1.5 Ma, with a marked increase in the divergence process during the Pliocene.

Of the eight partitions generated in the ABGD analysis, the three-species option yielded the highest statistical significance ($P = 0.007$; Figure 1b). On the other hand, the PTP analysis identified between 2 and 6 potential species; however, only the four species with support values close to 0.5 were considered. Finally, the GMYC clustering analysis identified only one evolutionary entity, with a likelihood ratio of 19.12 ($P = 7.04\text{e-}05$).

Ecological niche analyses using PCA indicate consistent ecological segregation across most clades, except for clades A and B, which show a smaller Mahalanobis distance between their environmental centroids (Figure 4). The overlap between clades is low to moderate, suggesting possible ecological differentiation (Figure 5). In contrast, clade A and clade B showed a partial differentiation with some overlap (D = 0.23). The comparisons of clades B–C and B–D showed the least separation, with overlap approaching 1 in both comparisons ($D = 0.92$). The analysis indicated no evidence of similarity greater than expected by chance ($P > 0.05$), with the exception of clades B vs. D (*P*

Table 1. Results of the similarity and ecological niche equivalence analyses and their interpretation. The asterisk (*) indicates a significant difference.

Comparison	Similarity (D) *P*-value	Equivalences (D) *P*-value	Interpretation
A vs B	(0.07) 0.45	(0.03) 0.16	Dissimilar, equivalent
A vs C	(0.01) 0.57	(0.005) 0.97	Dissimilar, equivalent
A vs D	(0) 1	(0) 1.00	Dissimilar, equivalent
B vs C	(0.10) 0.12	(0.07) 0.94	Dissimilar, equivalent
B vs D	(0.21) 0.03*	(0.14) 0.88	Similar, equivalent
C vs D	(0) 0.34	(0.06) 0.51	Dissimilar, equivalent

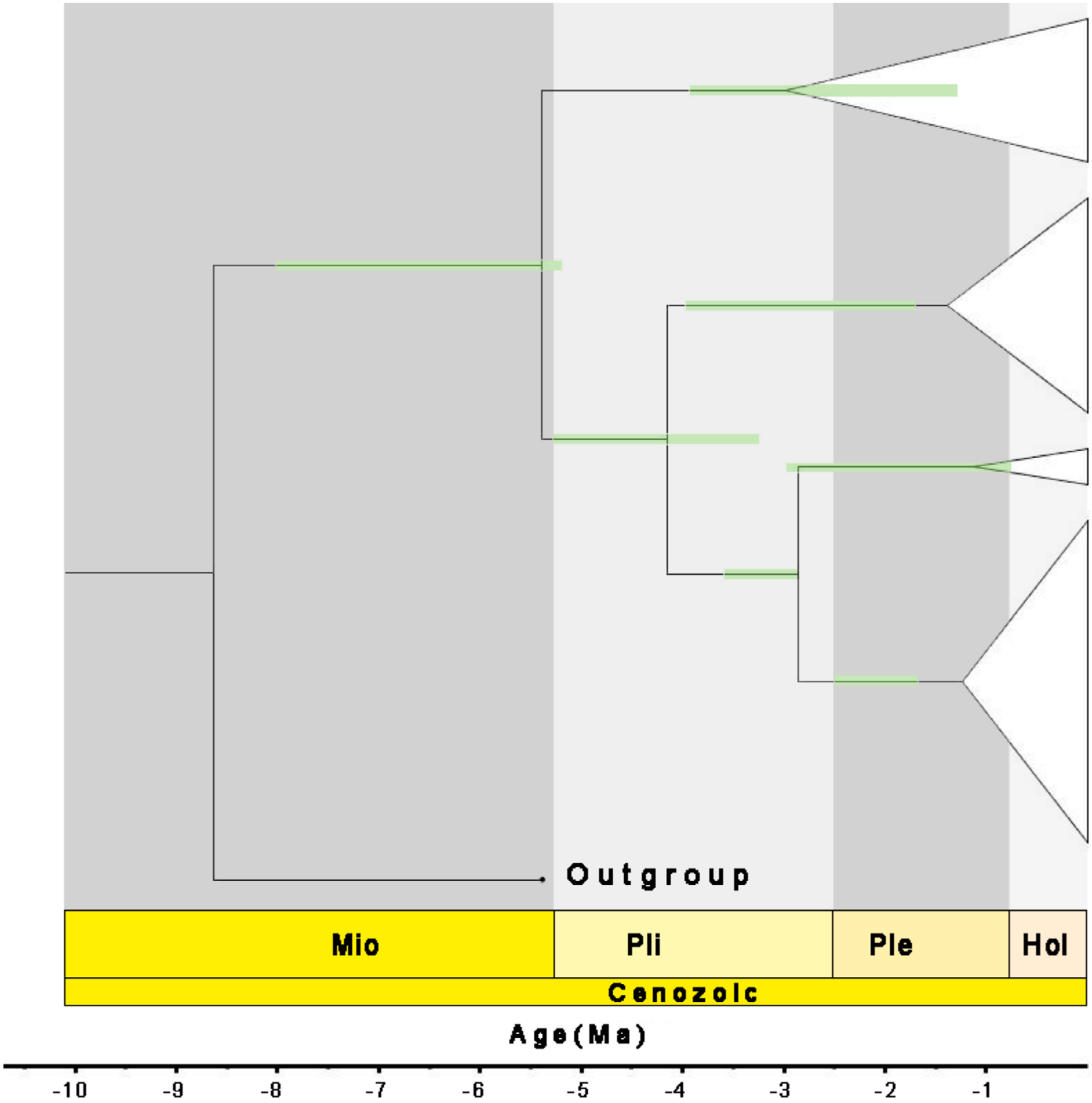


Figure 3. Phylogenetic relationships of the *Xerospermophilus spilosoma* complex calibrated with temporal divergence estimates. Nodes represent lineage-divergence events, and horizontal bars indicate 95% confidence intervals for estimated dates, scaled to the late Cenozoic era.

= 0.03; Table 1). On the other hand, in the equivalence test, none of the comparisons showed significant differences ($P < 0.05$), indicating that the niche models generated for each clade pair did not differ in their niches within the shared environmental space, with *D*-index values ranging from 0 to 0.14 (Table 1).

Discussion

The results suggest that *Xerospermophilus spilosoma* comprises a complex that hosts four evolutionarily independent lineages, a hypothesis supported by clear phylogenetic separation, consistent haplotype distributions across environmentally segregated populations, and differentiation of ecological niches among these lineages.

The estimated divergence times differ from those proposed by Fernández (2012), who suggested that the *X. spilosoma* complex is approximately 2.69 Ma old and that the last common ancestor of the San Luis Potosí and Perote lineages existed 0.74 Ma ago. In contrast, our results indicate that the *X. spilosoma* complex emerged toward the end of the Miocene, just over 5 Ma ago. Most divergence events are concentrated between 3.5 and 1.5 Ma and are consistent with the estimated ages of the geographical barriers that influenced their diversification.

Clade A is the first to diverge, possibly favored by the geographical barrier represented by the Rio Grande (Figure 6). This river, with a length of approximately 3,050 km, flows through a large portion of the southwestern United States, emptying into the Gulf of Mexico (Kelley 1952). It was formed during the Late Miocene and Pliocene between 6.9 and 2.5 Ma (Morgan and Golombek 1984; Gamez *et al.* 2017), consistent with the divergence time estimated in

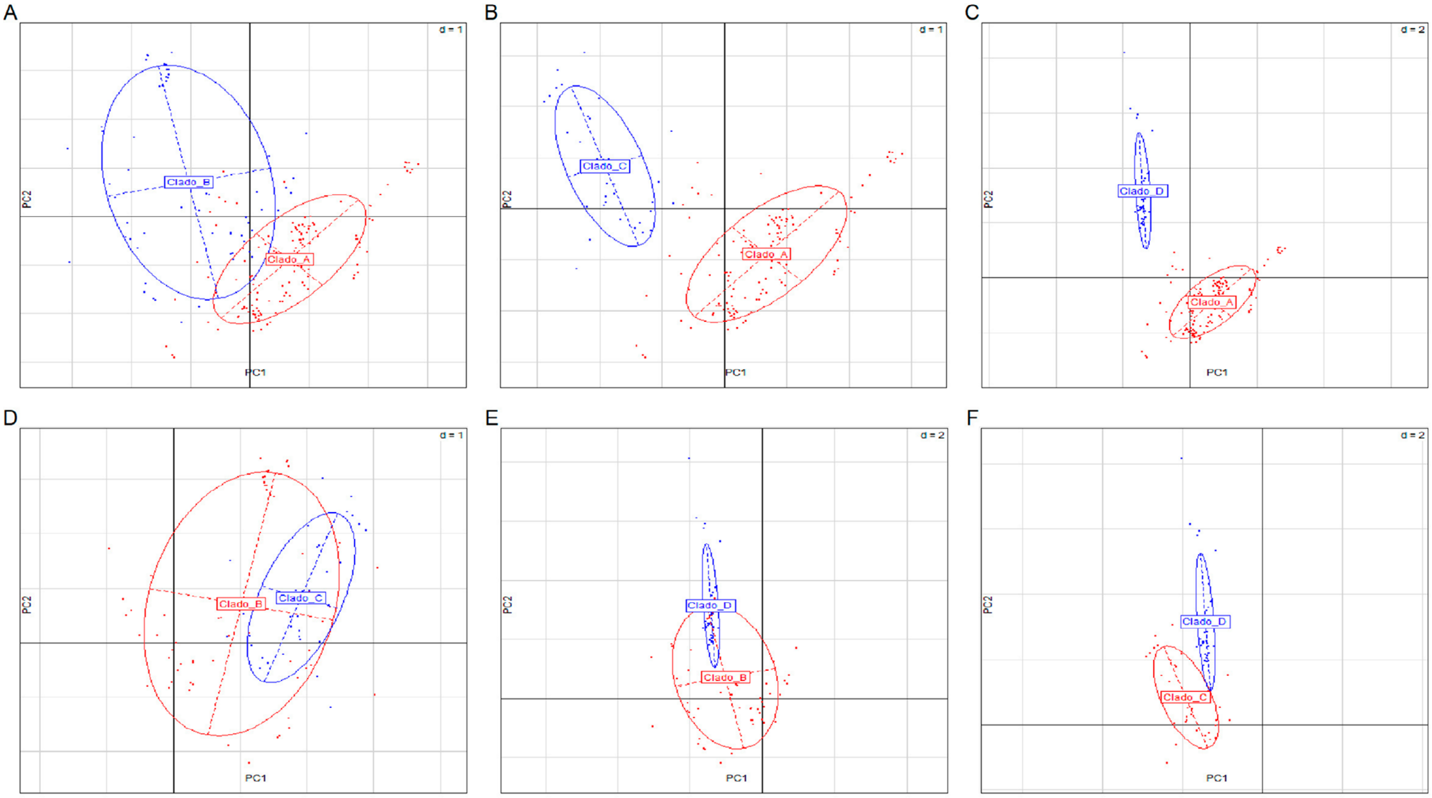


Figure 4. Ellipses indicate 95 % confidence areas around the means of each group; comparison between clades: A) A and B; B) A and C; C) A and D; D) B and C; E) B and D; and F) C and D.

this work. The importance of this water body as a driver of speciation processes has been documented for other rodents, such as *Chaetodipus* and *Eutamias* (Sullivan 1985; Neiswenter *et al.* 2019).

Clade B is distributed in the Trans-Pecos and Bolsón de Mapimí, while clade C is represented by the population inhabiting the Central Plateau in San Luis Potosí, Mexico. These three zones are subregions of the great Chihuahuan Desert, considered among the most diverse arid regions worldwide (Dinerstein *et al.* 2000; Edwards *et al.* 2001). The geological and climatic events of the Miocene and Pliocene, along with the cyclical climatic changes of the Pleistocene, had a profound effect on the genetic structure and distribution of various species that live in this desert (Raymo and Ruddiman 1992; Hafner and Riddle 2005; Loera *et al.* 2017; Scheinvar *et al.* 2020). These events possibly favored divergence between clades, which is supported by the divergence times estimated in this study, similar to the reports regarding the diversification between *Cynomys mexicanus* and *C. ludovicianus* (Castellanos-Morales *et al.* 2016), as well as about the genetic patterns of *Perognathus* and *Chaetodipus* mice (Riddle *et al.* 2000; Neiswenter and Riddle 2010) and the differentiated distributions of grasshopper mice of the genus *Onychomys* (Riddle 1995).

On the other hand, the divergence between clades B and C could be related to the Nazas River**,** whose formation is estimated to have occurred between mid- and late Pliocene (Petersen 1976; Hafner and Riddle 2011), a period estimated in the present work. This river has been identified as a possible biogeographic barrier for several species, including the rats *Neotoma albigula* and *N. leucodon* (Edwards *et al.* 2001), the cactus mouse *Peromyscus eremicus* (Riddle *et al.* 2000), gophers of the genus *Cratogeomys* (Hafner *et al.* 2008), and the black-tailed hare *Lepus californicus* (Lorenzo *et al.* 2021).

On the other hand, clade D is restricted to the Eastern Basin, a high-mountain region, formed mostly during the Pleistocene (<1.6 Ma) as part of the uplift of the Trans-Mexican Volcanic Belt (Ferrari *et al.* 1999). The emergence of this mountain range could have favored the divergence of this clade, as documented for other rodents, such as *Dipodomys phillipsi* and *Peromyscus bullatus* (Peterson *et al.* 2000; González-Ruíz *et al.* 2005; Sánchez-Cordero *et al.* 2005; Fernández *et al.* 2012).

The interspecific genetic distances observed within the *X. spilosoma* complex were slightly below the threshold proposed by Baker and Bradley (2006) to recognize entities as distinct species. However, these distances are similar to those accepted for other closely related genera, such as *Spermophilus*, when supported by additional evidence, such as genetic structure and multilocus analysis (Simonov *et al.* 2024). On the other hand, these divergence levels are consistent with evolutionarily independent lineages, as proposed for squirrel species of the genus *Tamias*, which supports their possible recognition as distinct species (Ge *et al.* 2014). The genetic diversity patterns found in *X. spilosoma*

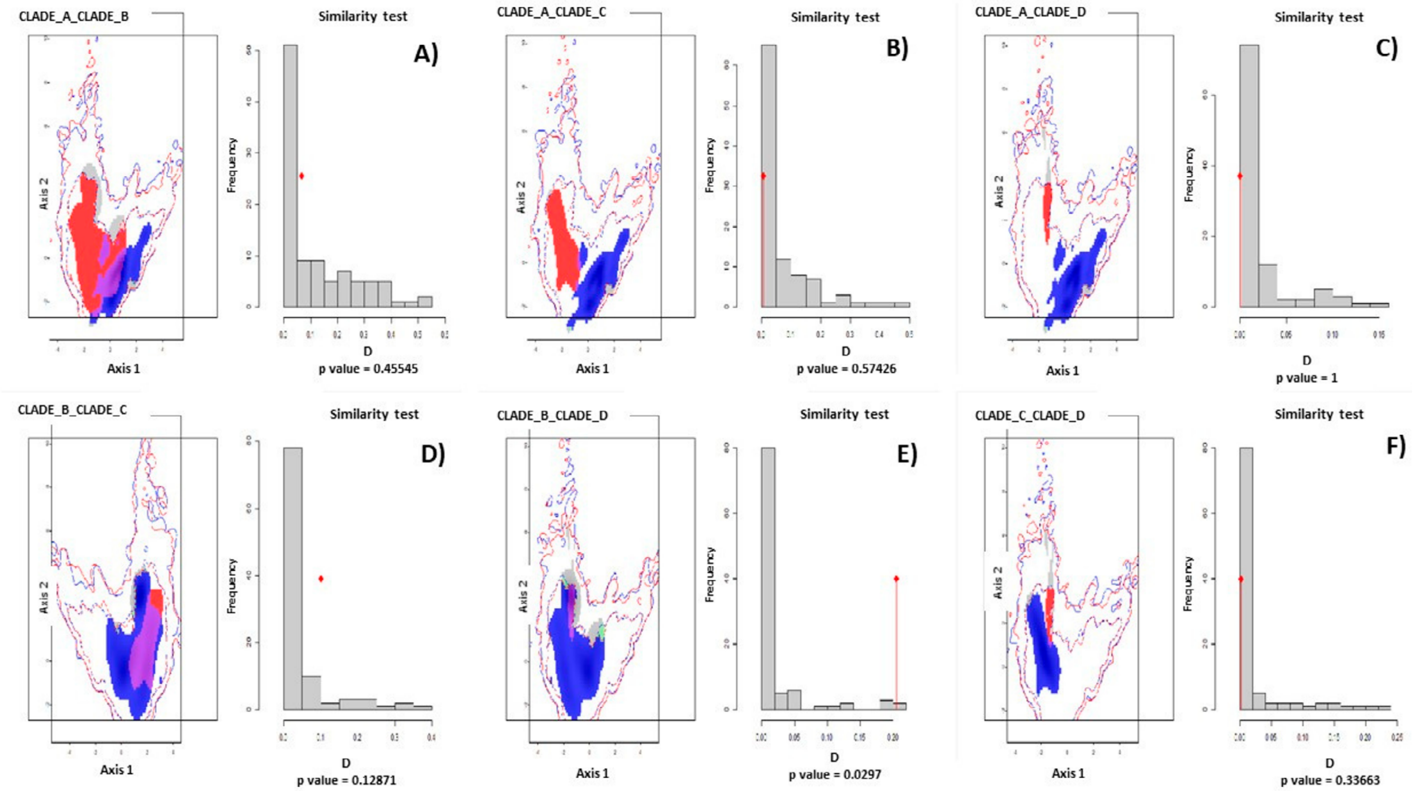


Figure 5. Comparisons between the environmental datasets of clades: A) A and B; B) A and C; C) A and D; D) B and C; E) B and D; and F) C and D, using a principal component analysis (PCA), showing the distribution of populations in the space defined by the first two principal components (PC1 and PC2). Bars in the similarity test histograms represent null models, and the red line represents the observed *D*-value.

in the present study reinforce this interpretation. A high Hd = 0.99 and a considerable Pi = 0.04 were observed, which are within the upper range reported for other rodents, such as *Neotoma mexicana* (Hd = 0.97; Pi 0.03–0.05) and *Reithrodontomys chrysopsis* (Hd = 0.99; Pi = 0.03) (Hernández-Canchola *et al.* 2021; León-Tapia *et al.* 2023).

On the other hand, the different species delimitation methods yielded inconsistent results, as they identified different numbers of evolutionary entities. Such discrepancies between methods are common, as each approach is based on different assumptions and models (Feijó *et al.* 2019; Martínez-Borrego *et al.* 2023). The partial concordance between ABGD and PTP suggests some degree of genetic structure within *X. spilosoma*, although the low resolution of GMYC could indicate recent diversification or insufficient mitochondrial differentiation, which warrants further analysis.

In addition, correspondence between genetic structure and ecological niche modeling was observed. Clades A, C, and D exhibited marked ecological differentiation, with minimal or no niche overlap among them. In contrast, we found evidence of partial or complete niche overlap between clade B and all other clades. This overlap can be explained by the niche breadth of clade B, associated with the marked climatic heterogeneity of the Mapimí region, which offers a wide range of environmental conditions (Van Devender and Burgess 1985; García-Arévalo and Nocedal 2008). Although the overall pattern is a positive relationship between niche breadth and geographic range (Morin and Lechowicz 2013; Slatyer *et al.* 2013; Moulatlet *et al.* 2025), niche breadth does not necessarily correspond to a larger potential distribution area, since the conditions that make up that niche may be unequally represented in geography (Dallas and Ten Caten 2025). For example, clade A has a potential distribution area of 637 867 km², more than twice the estimated area for clade B (309 468 km²), with a smaller environmental niche breadth; this highlights the importance of the environmental heterogeneity of the Mapimí region and is related to the distribution of clade B (García-Arévalo and Nocedal 2008).

Statistically, the ecological niche of clade B showed similarity only with the niche of clade D. Niche similarity between clades B and D could be explained by a non-typical niche phylogenetic conservatism, in which species maintain environmental similarities regardless of their genetic distances (Wiens and Graham 2005: Losos 2008). It is also possible that both clades are preserving a niche close to a midpoint within the environmental space occupied by the clade set (Wiens *et al.* 2010; Peixoto *et al.* 2017). However, the geographical distance between populations, genetic evidence, and the limited dispersal capacity of squirrels suggest that clades B and D are distinct groups despite their ecological proximity. The equivalence test was not significant, implying that the compared niches cannot be considered identical. This is consistent with the fact that the equivalence test is more conservative

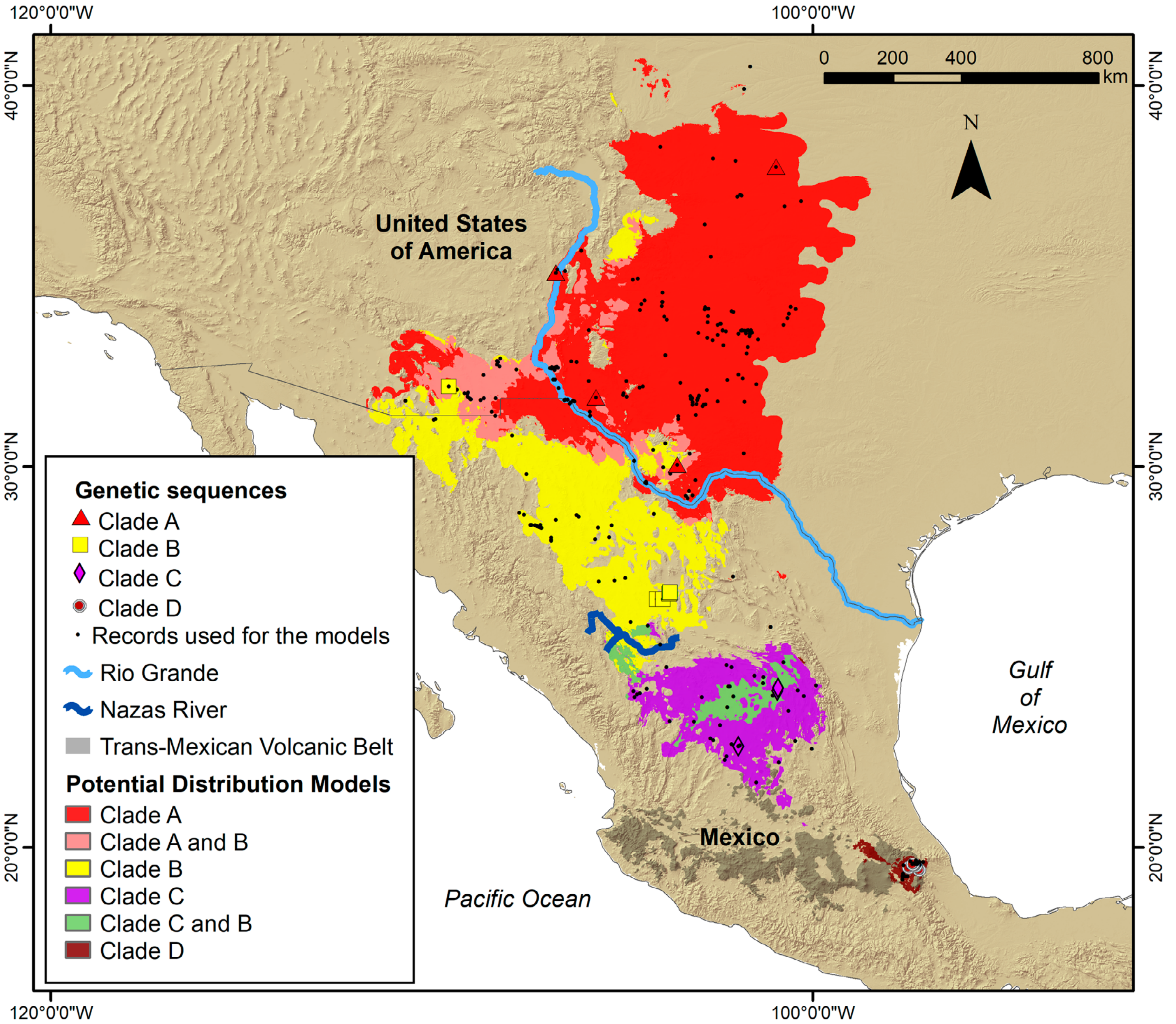


Figure 6. Potential distribution map of phylogenetic clades A, B, C, and D generated by ecological niche modeling using the MAXENT algorithm. The colored areas indicate the estimated environmental suitability for each clade, and symbols (triangle, square, rhombus, and circle) represent collection locations.

than the similarity test, as it assesses only whether two niches are indistinguishable from actual locations, without incorporating the surrounding environmental space (Brown and Carnaval 2019).

Taken together, the lines of evidence discussed here highlight the importance of considering both evolutionary history and local ecological conditions in order to understand diversification processes within the *X. spilosoma* complex. The evidence from the present study suggests that this complex can be divided into four lineages. Since species delimitation should not be based solely on absolute genetic distances, it is essential to integrate other factors such as monophyly, phylogenetic structure, and the gradual nature of allopatric speciation, the pace of which may vary according to the ecological, genetic, and geographical conditions involved (Carstens *et al.* 2013). In addition, it is important to recognize that genetic divergence can advance even in the absence of differentiated ecological selective pressure (Wiens 2004; Nosil 2012).

In the case of clade D (*X. perotensis*), the data support its recognition as an evolutionarily distinct lineage characterized by geographic isolation, a unique genetic structure, monophyly, and ecological differentiation from the other clades. These elements support its recognition as a sister species of *X. spilosoma* under the unified species concept framework. However, inconsistencies between the delimitation methods and the molecular evidence, limited to a mitochondrial gene fragment, preclude a definitive taxonomic conclusion. An alternative interpretation is that *X. perotensis* is at an advanced stage of the allopatric speciation continuum.

Although only a partial region (690 bp) of the *cytochrome b* gene was analyzed, our results provide solid evidence on genetic structuring and ecological differentiation in squirrels of the genus *Xerospermophyllus*. A more complete and robust reconstruction of the evolutionary history of this taxonomic complex warrants the incorporation of additional mitochondrial and nuclear markers, along with other types of evidence (e.g., morphological, behavioral, or genomic). It will also be essential to increase the sample size and include additional populations, such as those inhabiting the Great Tamaulipas Desert, which could not be included in this study due to insufficient data. Finally, a major limitation in the reconstruction of ecological niche models is the lack of biotic data, such as ecological interactions, resource availability, or predator pressure, which restricts the power of models to more accurately discriminate between ecological niches of different clades (Peng *et al.* 2025).

While the taxonomy of this squirrel complex is being resolved, it is important to recognize that the identification of genetically differentiated clades and distinct ecological niches underscores the need to conserve each genetic lineage as a significant evolutionary unit. This is particularly relevant for the Perote population, the southernmost and most isolated squirrel population, which, due to its distinctive characteristics, is essential for preserving the genetic and ecological diversity of this group.

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Appendix 1

Appendix 1. Geographical data of the specimens used and National Center for Biotechnology Information (NCBI) access number. In the species column, X = *Xerospermophilus*; I = *Ictydomys*; and C = *Cymomys*.

Species	**Country**	**State**	**Latitude**	**Longitude**	**Collection ID**	**NCBI #**	**References**
X spilosoma	USA	Kansas	37.872	-100.964		AF157885	Harrison *et al.* 2003
X spilosoma	USA	Kansas	35.084	-106.74		AF157911	Harrison *et al.* 2003
X spilosoma	USA	New Mexico	35.084	-106.738		JX047300	Fernández 2012
X spilosoma	USA	Arizona	32.1080556	-109.555278	ACUNHC 566	PX673950	This study
X spilosoma	USA	Texas	30.0487278	-103.551506	ACUNHC 1376	PX673951	This study
X spilosoma	USA	Texas	31.8174644	-105.688989	ACUNHC 2180	PX673952	This study
X spilosoma	Mexico	Durango	26.523	-104.089		AF157845	Harrison *et al.* 2003
X spilosoma	Mexico	Durango	26.524	-103.929		AF157846	Harrison *et al.* 2003
X spilosoma	Mexico	Durango	2953720	624503	AGR01	PX673953	This study
X spilosoma	Mexico	Durango	2950540	623514	AGR03	PX673954	This study
X spilosoma	Mexico	Durango	2953955	624710	UNAM EY1194	PX673955	This study
X spilosoma	Mexico	Durango	2953955	624710	UNAM MVA103	PX673956	This study
X spilosoma	Mexico	San Luis Potosí	24.125	-100.925		DQ106853	Chumacero *et al.* 2006
X spilosoma	Mexico	San Luis Potosí	24.2	-100.901667		DQ106854	Chumacero *et al.* 2006
X perotensis	Mexico	Puebla	19.49	-97.489		AF157840	Harrison *et al.* 2003
X perotensis	Mexico	Puebla	19.49	-97.489		AF157948	Harrison *et al.* 2003
X perotensis	Mexico	Veracruz	19.587	-97.33		JX047301	Fernández 2012
X perotensis	Mexico	Puebla	19.49	-97.489		JX047302	Fernández 2012
X perotensis	Mexico	Veracruz	19.572	-97.383		JX047303	Fernández 2012
X perotensis	Mexico	Veracruz	32.4166667	-97.8166667	AMS01	PX673957	This study
X perotensis	Mexico	Veracruz	2161070	678473	AMS02	PX673961	This study
X perotensis	Mexico	Veracruz	2161070	678473	AMS03	PX673960	This study
X perotensis	Mexico	Veracruz	2161070	678473	AMS04	PX673958	This study
X perotensis	Mexico	Veracruz	2161070	677627	AMS06	PX673959	This study
I mexicanus	Mexico	Edomex				AF157848	Harrison *et al.* 2003
C ludovicianus	Mexico	Chihuahua				JQ885590	Castellanos-Morales *et al.* 2014