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The low chromosome number of a relict shrew (*Cryptotis magnus*) isolated in a cloud forest of México

El bajo número cromosómico de una musaraña relicto (*Cryptotis magnus*) aislada en un bosque mesófilo de México

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To know and conserve Mexican shrews it is necessary to know them well, although their taxonomic identification is difficult. However, cytogenetic tools can help to discriminate species. There are no known karyotypes of Mexican shrews, so we decided to initiate the study of their chromosomes starting with *Cryptotis magnus*. Shrews were collected in the cloud forest of Oaxaca and chromosomal metaphases were prepared from bone marrow, stained with Giemsa, examined microscopically, and photographed. Chromosomes were examined and counted to calculate the 2*n*, determine their morphology, and estimate the FN; finally, a karyotype was prepared. Specimens of *C. magnus* were cataloged in the Colección Nacional de Mamíferos, Instituto de Biología, UNAM. Results showed numerous metaphase cells and conspicuous chromosomes. The diploid chromosome number found was 2*n* = 26, consisting of 24 biarmed autosomal chromosomes (18 metacentric and 6 submetacentric) and 2 sex chromosomes (X: large subtelocentric; Y: small telocentric). Adding the number of chromosome arms of the autosomes the resulting FN was 48. The chromosomal complement of *C. magnus* is distinctive and its 2*n* is one of the smallest in the family Soricidae. The current conformation of its chromosomal profile may have originated from Robertsonian chromosomal rearrangements that reduced the ancestral 2*n* (46) to 2*n* = 26 producing autosomes of biarmed morphology. Geographic isolation in a small geographic region of Oaxaca may have facilitated this evolutionary process.

Key words: Cytogenetics; Eulipotyphla; Evolution; karyotype; New World; Oaxaca; Soricidae.

Para conocer y conservar a las musarañas mexicanas es necesario conocerlas bien, aunque su identificación taxonómica es difícil. Sin embargo, las herramientas citogenéticas pueden ayudar a discriminar las especies. No se conocen cariotipos de musarañas de México, por lo que decidimos iniciar el estudio de sus cromosomas comenzando con *Cryptotis magnus*. Se colectaron musarañas en bosque de niebla de Oaxaca y se prepararon metafases cromosómicas de médula ósea, se tiñeron con Giemsa, se examinaron microscópicamente y se fotografiaron. Se contaron los cromosomas para calcular el 2*n*, se determinó su morfología, se estimó el FN y se elaboró un cariotipo. Los especímenes de *C. magnus* fueron catalogados en la Colección Nacional de Mamíferos, Instituto de Biología, UNAM. Los resultados mostraron numerosas células metafásicas y cromosomas conspicuos. El número cromosómico diploide encontrado fue de 2*n* = 26, formado por 24 cromosomas autosómicos birrámeos (18 metacéntricos y 6 submetacéntricos) y 2 cromosomas sexuales (X: subtelocéntrico grande; Y: telocéntrico pequeño). Sumando el número de brazos cromosómicos de los autosomas el número fundamental (FN) resultante es 48. El complemento cromosómico de *C. magnus* es distintivo y su 2*n* es uno de los más pequeños de la familia Soricidae. La conformación actual de su perfil cromosómico puede haberse originado a partir de reordenamientos cromosómicos Robertsonianos que redujeron el 2*n* ancestral (46) a 2*n* = 26 produciendo autosomas de morfología birrámea. El aislamiento geográfico en una pequeña región geográfica de Oaxaca puede haber facilitado este proceso evolutivo.

Palabras clave: Cariotipo; Citogenética; Eulipotyphla; Evolución; Nuevo Mundo; Oaxaca; Soricidae.

Shrews (Soricidae) are small mammals widely distributed in most terrestrial ecosystems in México, belong to one of the orders (Eulipotyphla) with the highest number of species (Sánchez-Cordero *et al.* 2014) and have phylogenetic importance as they conserve the morphotype of primitive mammals. In addition, they play an important ecological role as both predators of many invertebrates and prey of reptiles, birds, and mammals. Over half of Mexican shrew species are endemic and listed in some risk category (Guevara *et al.* 2015). Therefore, they are an important component of Mexican biodiversity that should be studied and protected. However, shrews are little studied since it is infrequent to observe them alive in their natural environment; but mainly because it is not easy to collect them in the field. Likewise, their taxonomic identification is problematic even for specialists, since most of them are usually highly similar in morphology.

Historically, cytogenetic approaches have been used as additional tools to complement the taxonomic identification of mammals (Genoways *et al.* 2020). This perspective provides a complementary view of the degree of chromosomal differentiation between taxa, allowing taxonomic boundaries and phylogenetic relationships to be hypothesized (Graphodatsky *et al.* 2011; Biltueva and Vorobieva 2012). To this end, the representation of the chromosomal pattern of a species or karyotype has proven to be a useful and important tool in characterizing cytogenetic attributes that help distinguish different species of mammals (Levan *et al.* 1964).

Unfortunately, the karyotype of only 3 of the 42 shrew species currently recognized for México is known, but no specimen examined so far has come from Mexican territory. Therefore, to begin to fill this gap in the cytogenetic knowledge of Mexican soricids, we studied the karyotype of the big Mexican small-eared shrew (*Cryptotis magnus*), a member of the *C. mexicanus* species group characterized by its relatively large size compared to other *Cryptotis* from North America (He *et al.* 2021). It has a very restricted distribution in the north-central part of the state of Oaxaca, in southern México, and inhabits the cloud forest between 1,200 and 3,000 m (Carraway 2007; Guevara *et al.* 2015). Its long tail and primitive cranial and dental characteristics would seem to indicate that *C. magnus* is the only surviving representative of an ancient lineage (Choate 1970). The Mexican government and the IUCN classify this small mammal in the risk categories "under special protection" and "vulnerable", respectively (Cuarón and de Grammont 2018; Trujillo Segura 2019).

Specimens of shrews were collected in the vicinity of the village Santa Catarina Ixtepeji (17° 13' 18" N, 96° 35' 2" W), municipality Santa Catarina Ixtepeji, Oaxaca, México, at 2,304 m (Figure 1). The climate is temperate sub-humid (Instituto Nacional de Estadística y Geografía; INEGI 2013) with an average annual temperature of 12.8 °C and average annual precipitation of 1,299 mm (Fick and Hijmans 2017). The typical vegetation of the place is cloud forest characterized by the presence of the oak *Quercus* aff. *laurina* (Zacarías-Eslava and del Castillo 2010).

Shrews were trapped using pitfall traps (Martin *et al.* 2011) placed in patches of preserved forest and prepared in a conventional way for scientific study (Hall 1981). We

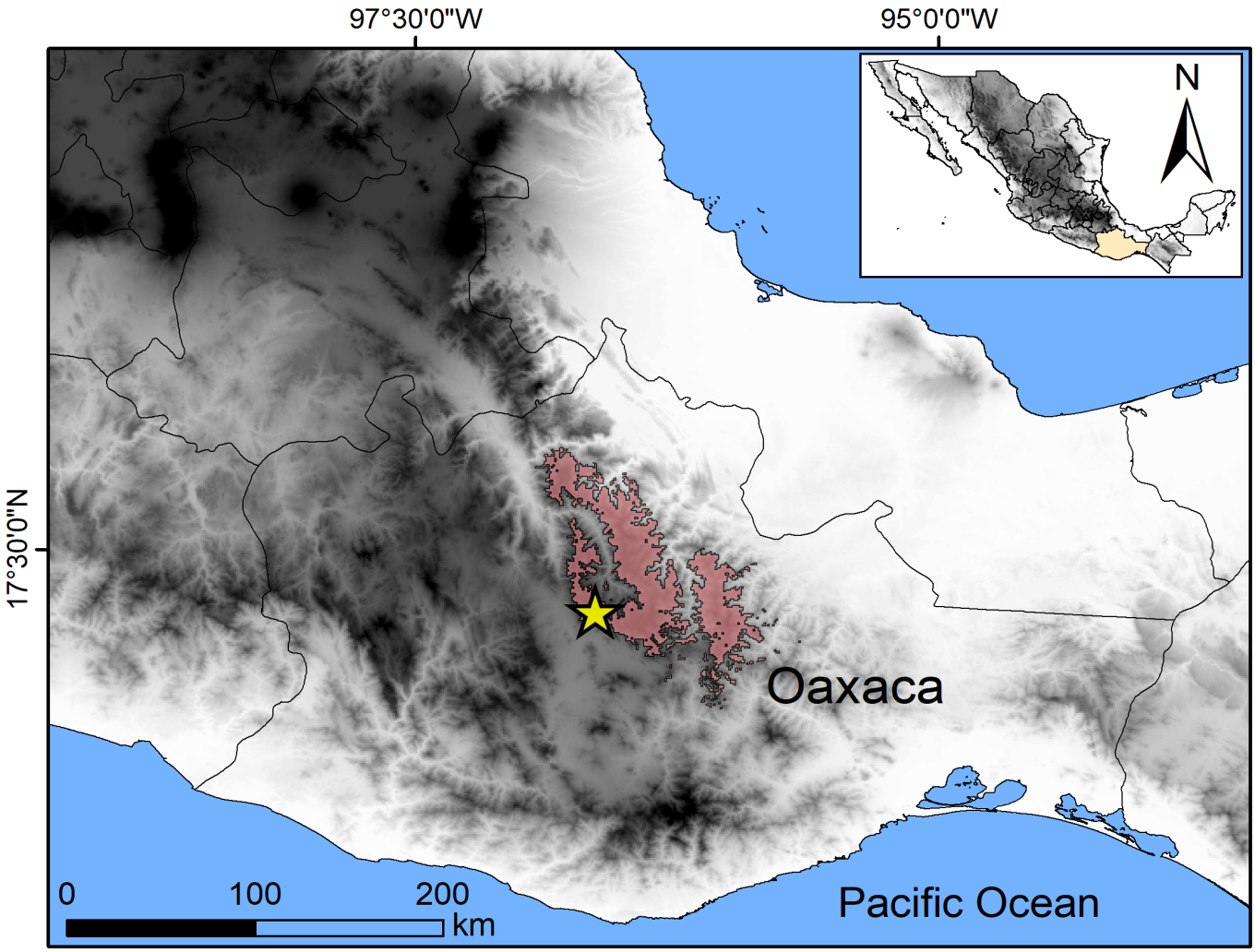

Figure 1. Potential geographic distribution of the big Mexican small-eared shrew (*Cryptotis magnus*; modified from Sánchez-Cordero *et al.* 2013). The yellow star indicates the site where specimens to prepare karyotypes were collected.

followed conventional guidelines for using wild mammals in research (Sikes *et al.* 2016) and regulations of a collecting permit of the Mexican government (FAUT-0002). Taxonomic identity of the specimens was confirmed using identification guides by Carraway (2007) and Álvarez-Castañeda *et al.* (2017).

Metaphase chromosomes of the shrews were obtained from bone marrow cells following procedures by Patton (1967) and Baker *et al.* (2003) with modifications. The entire procedure for obtaining cell samples was performed in the field.

Briefly, cell samples from each specimen were extracted from bone marrow perfused from the femur with an insulin syringe (27G x 13 mm) and 3 ml of RPMI-1640 transport medium (Sigma-Aldrich); the mixture was collected in a sterile 15 ml centrifuge tube (Eppendorf) and vigorously mixed to homogenize; 0.2 ml colchicine (Sigma, 0.05 µg/ml final concentration) was added, mixed again and incubated at 37 °C for 45 min, after which 5 ml of hypotonic solution (0.05 M KCl, Merck) was added, and incubated again at 37 °C for 30 min. After that, 1 ml of cold fixative solution (methanol / glacial acetic acid, 3:1 v/v, JT Baker) was added to the suspension for prefixation; the blend was homogenized and allowed to stand for 3 min. The sample was then centrifuged for 5 min at 1,000 rpm in a manual centrifuge; the supernatant was removed, and the blend was re-suspended. Finally, 5 ml of fixative solution was added, and the mixture was homogenized. At last, slides were prepared to obtain chromosomal metaphases conventionally stained with 2.5 % Giemsa (Sigma; Baker and Qumsiyeh 1988).

Chromosomes were searched for in the slides with a Zeiss Axioskop optical microscope at 10X and 40X and photographed at 100X with a Zeiss Axiocam Erc5s digital camera and the Zen lite software. Counts were made from the photographs to determine the diploid chromosome number (2*n*) and the morphological characterization (long arm and short arm measurements) of the chromosomes to classify them and determine the fundamental number (FN); then, a karyotype was elaborated based on the position of the centromere and size of the chromosomes (Levan *et al.* 1964; Baker and Hafner 1994).

Specimens of *C. magnus* from which the karyotype was obtained were cataloged in the National Collection of Mammals (CNMA) of the Institute of Biology, National Autonomous University of México as follows: CNMA49304-49307, 9.3 km SW Santa Catarina Ixtepeji; CNMA49308-49309, 9.7 km SW Santa Catarina Ixtepeji; CNMA49310-49312, 10.7 km SW Santa Catarina Ixtepeji; CNMA49313, 11.5 km SW Santa Catarina Ixtepeji; CNMA49314-49316, 11.8 km SW Santa Catarina Ixtepeji.

We obtained many metaphase cells from the cell samples of *C. magnus*. Eight metaphases useful for chromosome counting were selected as they showed acceptably separated and dispersed chromosomes (Figure 2a). The total number of chromosomes per metaphase counted for this species was 24 autosomes (non-sex chromosomes) and two sex chromosomes (X and Y), thus, its 2*n* = 26. All autosomes are biarmed (with 2 chromosome arms each), of which 18 are metacentric and 6 submetacentric. Therefore, the autosomal fundamental number (FN), or summation of all chromosome arms of the autosomes, is 48. The sex chromosome pair comprises a large biarmed subtelocentric and a small uniarmed telocentric (with only one chromosome arm; pair 13; Figure 2b). Of the 26 chromosomes, the longest length was 8.7 µm and that of the smallest was 1.5 µm.

Although there is scant comparative information in the literature, the chromosomal complement of *C. magnus* is quite distinctive, including that its 2*n* (26) is one of the lowest known so far (Table 1). The only other known cytogenetic study in this genus of soricids concerns a specimen of *C. parvus* from central Texas (Genoways *et al.* 1977). Its reported 2*n* was 52, twice that of *C. magnus*, the low chromosome number of a relict shrew (*C. magnus*) isolated in a cloud forest of México with all autosomes of the uniarmed

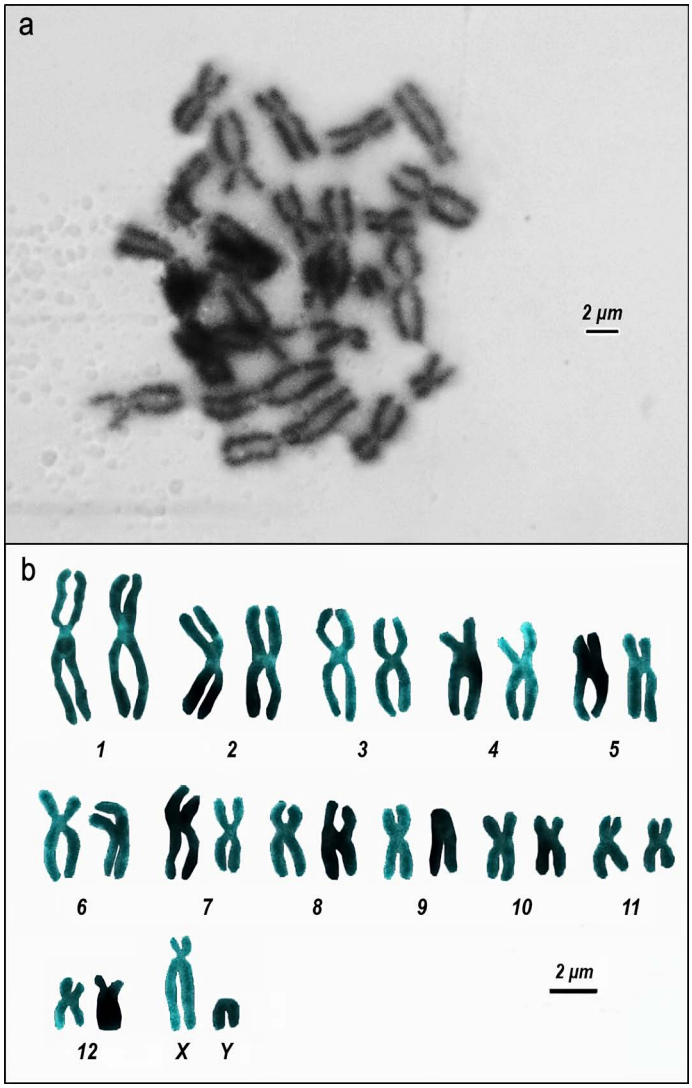

Figure 2. Chromosomes of a male big Mexican small-eared shrew (*Cryptotis magnus*; CNMA_ 49306). a) Metaphase viewed photomicroscopically at 100x. b) Karyotype (2*n* = 26 and FN = 48) showing chromosome morphology of the 24 biarmed autosomes and the sex chromosome pair (X and Y).

Table 1. Diploid chromosome number (2*n*) and fundamental number (FN) of shrew species (Eulipotyphla, Soricidae), and geographical area from which the reported karyotype was obtained.

Species	2*n*	FN	México	USA	Old World	Literature source
Cryptotis magnus	26	48	X			Present study
Cryptotis parvus	52	-		X		Genoways *et al*. (1977)
Sorex ornatus	54	-		X		Owen and Hoffmann (1983)
Sorex monticolus	54	-		X		Smith and Belk (1996)
Notiosorex crawfordi	34	38		X		Baker and Hsu (1970)
Sorex cinereus	66	70		X		Volobouev and Jong (1994)
Sorex haydeni	64	66		X		Volobouev and Jong (1994)
Sorex merriami	32	34		X		Rausch and Rausch (2004)
Sorex pacificus	54	58-59		X		Carraway (1985)
Sorex trowbridgi	34	38		X		Rausch and Rausch (2004)
Sorex vagrans	54	58		X		Owen and Hoffmann (1983)
Blarina hylophaga	52	60-62		X		George *et al*. (1982)
Blarina brevicauda	48-50	48		X		George *et al*. (1982)
Blarina carolinensis	34-52	41-62		X		George *et al*. (1982)
Sorex alpinus	56	54			X	Lukacova *et al*. (1996)
Sorex granarius	36-37	-			X	Biltueva and Vorobieva (2012)
Sorex araneus	20-50	-			X	Biltueva and Vorobieva (2012); Wójcik *et al*. (2003)
Blarinella griselda	44	-			X	Biltueva and Vorobieva (2012)
Crocidura watasei	26	56			X	Biltueva *et al*. (2001)
Crocidura dsinezumi	40	56			X	Biltueva *et al*. (2001)
Crocidura suaveolens	40	50			X	Biltueva *et al*. (2001)
Suncus murinus	40	56			X	Biltueva *et al*. (2001)

type, whereas those of *C. magnus* are all biarmed. Yet, the variation in autosome size in *C. parvus* (from 1 large pair to several small pairs) is proportionally greater than that observed in *C. magnus*. Also, the sex chromosomes of these species show some minor differences. In contrast to *C. magnus*, *C. parvus* has a large metacentric X chromosome and a small subtelocentric chromosome.

The karyotype of *Blarina*, sister genus to *Cryptotis* in the tribe Blarinini (Woodman 2018), also shows similar differences with *C. magnus*. *Blarina brevicauda* in Nebraska and Pennsylvania has a 2*n* of 49 or 50 (Genoways *et al.* 1977), whereas *B. carolinensis* in Nebraska and Kansas has a 2*n* of 52. In both species, the X chromosome is also a large metacentric and the Y chromosome is a small acrocentric. The FN of the above 3 species (*C. parvus* = 50, *B. brevicauda* = 48, and *B. carolinensis* = 62) are distinct due to their differences in the number of biarmed and uniarmed elements.

As to the cytogenetics of the 5 shrew species of the tribe Notiosoricini (genera *Megasorex* and *Notiosorex*), endemic to México and the USA, only *N. crawfordi* is known to have a 2*n* of 68 (FN = 102) and 62 (FN = 94), in Texas and Arizona, respectively (Baker and Hsu 1970). These diploid chromosome numbers also double that reported here for *C. magnus*.

The shrew genus *Sorex* is holarctic in distribution. Of the 49 New World species of *Sorex* (Woodman 2018), 16 inhabit México (Guevara *et al.* 2015), of which only the 2*n* (= 54) of *S. ornatus* and *S. monticola* are known (Table 1); however, the reported specimens correspond only to American localities. On the other hand, it is interesting that *S. merriami* and *S. trowbridgii* have 2*n* = 32 and 34, respectively, numbers close to, but even higher than, those of *C. magnus*.

On the other hand, the 2*n* of *C. magnus* is far from the ancestral mammalian karyotype of 2*n* = 46 proposed by Ferguson-Smith y Trifonov (2007). Therefore, several chromosomal rearrangements were required to reduce its 2*n* to 26. Chromosome numbers can increase or decrease by chromosome fission or fusion (Robertsonian events), respectively (Zima 2000), as has been documented in the chromosomal evolution of Eulipotyphla (Biltueva and Vorobieva 2012), suggesting that chromosomal variation may contribute to speciation. *Cryptotis magnus* had been considered a relict species geographically isolated in a relatively small region of the cloud forest of northern Oaxaca, México (Choate 1970). However, its karyotype suggests a chromosomal set with a remarkable number of changes. Whether centromeric fusion events in the chromosomal complement of *C. magnus* resulted in a shrew species with one of the lowest known chromosomal numbers accompanied of biarmed autosomes after geographic isolation remains to be investigated.

Our research is the first cytogenetic study of a shrew species from Mexican territory. We hope this result will promote research on the karyotypes of the other 41 recognized species of Mexican shrews to know and interpret the intra- and interspecific similarities and differences among soricid species of the New World. Chromosomal studies are still an important tool to record and describe biological diversity and often represent a simple and indispensable method for identifying various taxa (Zima 2000; Biltueva and Vorobieva 2012), and the elucidation of their phylogenetic relationships (Biltueva *et al.* 2001).

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