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Filogenia y Evolución molecular del orden Scorpiones en México

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P R E S E N T A

M. en C. Sonia Dávila Ramos

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M. en C. SONIA DAVILA RAMOS
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A mis padres que me siguen acompañando en mis sueños

A mi familia por la cual me siento inmensamente afortunada

A mis amores Humberto y Valeria

INDICE

ABREVIATURAS

aa	aminoácido
AB	Análisis Bayesiano
C	Centruroides
cDNA	DNA complementario al RNA
cox1-3	genes de citocromo oxidasa subunidades I-III (COI-III, produto protéico)
DNAmt	DNA mitocondrial
ML	Máxima Verosimilitud (Maximum Likelihood)
MP	Máxima Parsimonia
ORF	Marco de lectura abierto ("Open Reading Frame")
pb	Pares de bases
PCR	Reacción en cadena de la polimerasa ("Polimerase chain reaction")
rrnS y rrnL	genes para la subunidad pequeña y grande del RNAribosomal (productos, s-rRNA y l-rRNA)
trnX	genes para RNA de transferencia, con la letra correspondiente al aminoácido en el código de una letra
tRNA Xxx	RNAs de transferencia con las letras correspondientes al aminoácido en el código de tres letras

LISTA DE FIGURAS

RESUMEN

Los alacranes son los artrópodos más antiguos que se conocen, por lo que ocupan un lugar importante en la interpretación de la filogenia de Chelicerata. El obtener la secuencia completa del genoma mitocondrial de un alacrán, nos permite contribuir al acervo genómico dentro de este grupo, que a la fecha incluye 17 genomas mitocondriales completos. Además de que proporciona la posibilidad de seleccionar distintos marcadores moleculares para resolver las relaciones filogenéticas dentro de los Chelicerata. A pesar de que los alacranes son un grupo relativamente bien estudiado debido a su importancia médica, poca atención se ha prestado al aspecto taxonómico. En América del norte todas las especies consideradas peligrosas para los humanos pertenecen al género *Centruroides*, es por esto que existe un gran interés por conocer tanto, los aspectos taxonómicos que permiten su clasificación, así como, las relaciones filogenéticas mantenidas entre las especies.

El género *Centruroides* esta compuesto de 46 especies, las cuales se distribuyen en México, el Caribe, Centro América, la parte sur de Estados Unidos y la parte Norte de América del Sur. La mayor parte de las especies utilizadas en este estudio provienen de México. Los primeros análisis filogenéticos realizados en este trabajo se obtuvieron con la construcción de una matriz de caracteres morfológicos y morfométricos. Estos análisis permiten comparar organismos que son difíciles de obtener en el campo debido a su poca abundancia y limitada distribución y hacer uso de las colecciones de algunas universidades o museos. Aunque este método presenta ciertas ventajas es bien conocido que los alacranes han sido capaces de resistir cambios geológicos permaneciendo casi inalterados morfológicamente por lo que es obvio que la cantidad de caracteres que pueden ser utilizados es mucho menor a la que se puede obtener de datos moleculares. El desarrollo de algunas técnicas moleculares en las últimas décadas han favorecido el estudio filogenético y evolutivo de muchas especies, esto permite la comparación de una mayor cantidad de caracteres a partir de marcadores moleculares determinados. En este trabajo fueron seleccionados dos genes mitocondriales (COI y COII) para determinar las relaciones filogenéticas entre las especies del género *Centruroides*. La utilización de datos morfológicos y moleculares, así como de distintos métodos de reconstrucción filogenética, nos ayuda a entender mejor las relaciones filogenéticas dentro del género *Centruroides*.

SUMMARY

The scorpions are the older arthropods known. That is why they play an important role in the interpretation of the phylogeny in Chelicerata. To obtain the complete mitochondrial sequence of scorpion, contributes to the genomic heap of all the Chelicerata, which to date includes 17 complete mitochondrial genomes and also makes the posibility to select different molecular markers to solve the phylogenetic relationships within Chelicerata.

Although the scorpions are relatively a well-studied group due to their medical importance, little attention has been given to their systematics. In North America all the species considered dangerous for the humans belong to the *Centruroides* genus. This increases the interest to know more about the taxonomic aspects to favor their classification and the phylogenetic relationships between the species.

The genus *Centruroides* contains 46 species, which are distributed in Mexico, the Caribbean, Central America, the Southern part of the United States and the Northern part of South-America. Most of the species (17 out of 19) used in this study comes from Mexico.

The first phylogenetic analyses made in this work were obtained using traditional taxonomy with the construction of a morphologic and morphometric characters matrix. These analyses allow to compare organisms from collections of the universities or museums that are difficult to obtain in the field due to their little abundance and limited distribution. Although this method has advantages, it is well known that the scorpions have been able to survive with few morphological changes over the geological time. Because of this, the informative morphological characters are smaller than the number that can be obtained from molecular data.

The development of some molecular techniques in the last decade has favored the phylogenetic and evolutionary studies of many species. This allows the comparison of a greater amount of characters from the selected molecular markers. In this work two mitochondrial genes were selected (COI and COII) to determine the phylogenetic relationships between the species of the *Centruroides* genus.

The use of morphologic and molecular data, as well as different methods of phylogenetic reconstruction, provide us a better understanding of the phylogenetic relationships within the *Centruroides* genus.

PRESENTACIÓN

En esta tesis se abordan algunos temas relacionados con la filogenia y evolución de los alacranes a distintos niveles jerárquicos. En primer lugar, basándose en el análisis del genoma mitocondrial, se observan las relaciones filogenéticas que guardan los alacranes con respecto a los demás órdenes pertenecientes a la clase Arachnida.

En segundo lugar, tomando en cuenta tanto datos morfológicos como moleculares, se sugieren las relaciones filogenéticas dentro del género *Centruroides*. Siendo éste uno de los grupos que mayor interés despiertan sobre todo en nuestro país, ya que anualmente se reportan muertes ocasionadas por la picadura de las especies más tóxicas del género.

La presente tesis está dividida en 7 capítulos.

El primero contiene una introducción a la biología general de los alacranes, haciendo hincapié en la sistemática, así como en los principales métodos de reconstrucción filogenética. También se incluyen los antecedentes sobre la filogenia de los Chelicerata y de la sistemática y clasificación de los alacranes del género *Centruroides*. Por último se presenta la hipótesis del trabajo así como los objetivos planteados para su realización.

El segundo capítulo está formado por el artículo titulado "The mitochondrial genome sequence of the scorpion *Centruroides limpidus* (Karsch 1879) (Chelicerata; Arachnida)", publicado en la revista "Gene" así como un comentario general del contenido del artículo.

El tercer capítulo incluye el artículo titulado "Phylogenetic analysis of scorpions from the genus *Centruroides* Marx, 1890 of Mexico, using morphological and morphometric characters" (sometido en el Acta Zoológica Mexicana), haciendo hincapié sobre las principales conclusiones obtenidas.

El cuarto capítulo presenta el artículo sometido a Molecular Phylogenetics and Evolution, que lleva por título "Phylogenetic Analysis of Genus *Centruroides* Based on Mitochondrial Cytochrome Oxidase I and II Genes", incluyendo el comentario sobre los puntos sobresalientes del artículo.

El quinto capítulo muestra algunas de las utilidades del análisis filogenético mediante el artículo publicado en la revista "Biochemie", titulado "Biochemical, genetic and physiological characterization of venom components from two species of scorpions: *Centruroides exilicauda* Wood and *Centruroides sculpturatus* Ewing" aunando las principales conclusiones del trabajo.

El sexto capítulo consta de una discusión general de los resultados obtenidos mostrados en los artículos publicados y sometidos.

Por último, en el séptimo capítulo, presentamos las conclusiones y perspectivas del trabajo.

CAPITULO I

INTRODUCCION

Origen y Evolución de los alacranes

Dentro de los Artrópodos, los alacranes son considerados los organismos más antiguos que se conocen y los más primitivos en su estructura corporal. Los fósiles de los primeros alacranes datan del periodo Silúrico (438-408 MYA), todos ellos presentan agallas bien desarrolladas, lo que indica que vivían en ambientes acuáticos (Brownell y Polis, 2001).

Estos primeros alacranes compartían muchas características con los euriptéridos, como la tagmosis, la segmentación, los apéndices, la presencia de opérculos branquiales y la existencia de ojos compuestos laterales, entre otras. Sin embargo, existen algunas divergencias evolutivas incluso con los alacranes más tempranos. La primera plantea que los alacranes carecían de válvulas anales, y la segunda muestra cierta diversidad en los apéndices respiratorios; como en el caso de los filamentos branquiales largos, característicos del proscorpioid del Devónico (408-360 MYA) (*Waeringoscorpio hefieri,* suborden Palaeoscorpinina) descrito por Stormer en 1970. Aún tomando en cuenta estas características, no es posible descartar la propuesta de que los alacranes se ramificaran a partir de un grupo de euriptéridos (Mixopteracean), en algún momento del Silúrico temprano (Figura 1) (Kjellesvig-Waering, 1986).

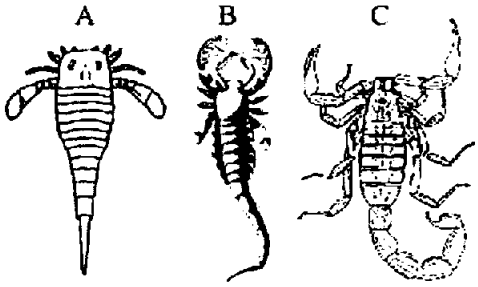

Figura 1 Esquema estructural de Euryptetida (A), Mixopteracean (B) y Scorpiones (C). Tomado de Stockwell, S. A. (página web), Polis, G. A., 1990.

La estructura corporal básica de los alacranes ha permanecido casi inalterada hasta nuestros días (Briggs, 1987). Tanto fósiles como alacranes actuales comparten caractrerísticas comunes: la presencia de peines (estructuras sensoriales), la existencia de las glándulas de veneno en el telsón y el desarrollo de pedipalpos fuertes y quelados. Estas autapomorfías (caracteres derivados) apoyan la monofília de los alacranes (Polis, 1990).

En el Devónico temprano se registran las primeras modificaciones de la gnatobase (estructuras para masticar); las cuales aparecen como una adaptación para la digestión externa en el hábitat terrestre. Los fósiles del género *Branchioscorpio* son considerados el tipo ancestral de transición de donde los alacranes terrestres provienen (Kjellesvij-Waering, 1986), ya que son los primeros en presentar las modificaciones anteriormente descritas y mantener el sistema respiratorio primitivo óptimo para vivir en el ambiente acuático.

Los alacranes y euriptéridos coexistieron y evolucionaron de manera paralela a través del Paleozoico medio y tardío. El registro fósil muestra que los alacranes estuvieron inmersos en un proceso de radiación, al explorar el ambiente terrestre, y que fueron los pequeños con patas fuertes los mejor preadaptados (Stockwell, 1989). No existe evidencia de que los alacranes acuáticos, que llegaron a medir hasta un metro (e. j. *Brontoscorpio*), ni los euriptéridos, exploraran el hábitat terrestre, quizás por su tendencia al gigantismo (Brownell y Polis, 2001).

Durante el periodo Carbonífero (360-286 MYA) aparecieron los primeros alacranes completamente terrestres. Los cambios más sobresalientes en el proceso de terrestrialización fueron: el engrosamiento de las patas por el aumento de peso, la sustitución de las agallas abdominales por pulmones de libro, y el incremento del espacio cercano a la boca, para favorecer la digestión externa y a su vez la alimentación líquida (Brownell, y Polis, 2001). Los alacranes branquiados persistieron hasta el periodo Jurásico (213-144 MYA).

Estructura corporal de los alacranes

El cuerpo de los alacranes está dividido en el prosoma o cefalotórax y el opistosoma o abdomen. Este último se encuentra conformado por el mesosoma en la parte anterior y el metasoma o cauda en la parte posterior (Figura 2).

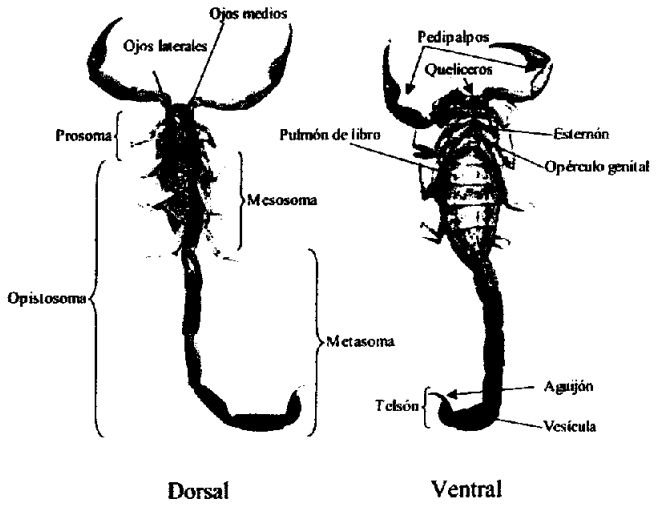

Figura 2 Morfología general de los alacranes.

En 1990, Hjelle concluyó que el cuerpo del alacrán consta de 21 segmentos. Autores previos han reportado variaciones a este número, debido a que algunos segmentos emergen o se pierden durante el desarrollo, como es el caso de los segmentos I y II del prosoma (Millot, y Vachon, 1949; Keegan, 1980).

El prosoma de un alacrán adulto consiste de 7 segmentos (III al IX), en los cuales se encuentran los músculos asociados a la locomoción y 6 pares de apéndices postorales (queliceros, pedipalpos y 4 pares de patas), cubiertos dorsalmente por la coraza del cefalotórax o carapacho. El carapacho contiene en la parte media un par de ojos (tubérculo ocular) y en su parte antero-lateral de 2 a 5 pares (Brownell, y Polis, 2001).

El mesosoma está constituido por 7 segmentos (X al XVI), cubiertos por una capa tergal esclerotizada, en la cual podemos localizar las estructuras reproductivas, los pulmones de libro, el corazón y la glándula digestiva. El metasoma consta de 5 segmentos (XVII al XXI) totalmente esclerotizados en su circunferencia; el ano se encuentra en la parte ventro-posterior del segmento XXI. En su parte distal se ubica el telsón o aguijón, el cual contiene las glándulas productoras de veneno y no es considerado como un segmento verdadero (Hjelle, 1990).

Distribución y clasificación de los alacranes

El evento más sobresaliente de la era Paleozoica (505-248MYA) fue el surgimiento del supercontinente llamado Pangea (Scotese, 1997). Los alacranes que habían colonizado el ambiente terrestre fueron capaces de dispersarse ampliamente, por lo que pudieron ocupar la mayoría de los continentes antes de su separación en el Mesozoico temprano. Una gran parte de los fósiles del Paleozoico y Carbonífero proviene de Norte América y Europa, regiones que se encontraban en Laurasia, la parte norte de la Pangea. Esto sugiere que las barreras marinas de la pre-Pangea pudieron intervenir en la distribución de estos y otros animales (Rolfe, 1982; Kjellesvij-Waering, 1986; Sissom, 1990).

Se considera que la mayoría de las familias tuvo su origen y distribución a partir de la región nombrada Godwana; los Chaerilidae se originaron en Laurasia y quedaron aislados en el sur de Asia, y los Buthidae se originaron en Laurasia pero se dipersaron hacia Godwana (Fet, y cols., 1999).

Actualmente los alacranes se encuentran en todos los continentes a excepción de la Antártida, y prácticamente ocupan todos los hábitats, desde desiertos, pasando por bosques e incluso en montañas cubiertas de nieve con elevaciones de hasta 5,500 metros. En algunos hábitats, la densidad de los alacranes puede ser de hasta 12 por metro cuadrado (Polis, 1990). En un hábitat ideal se pueden encontrar hasta 13 especies distribuidas simpátricamente, y en la mayoría de las áreas es común encontrar de 3 a 6 especies (Polis, 1990). La escasa variabilidad morfológica de los alacranes se contrapone a la gran plasticidad presente en su fisiología, comportamiento y respuesta al estrés ambiental; adaptabilidad que quizás haya favorecido su persistencia a lo largo del tiempo.

La clasificación de los alacranes ha cambiado significativamente en los últimos años. Los especialistas ahora reconocen más del doble de familias de alacranes de las 9 aceptadas en 1990. (Fet, y cols., 2000).

Actualmente se reconocen entre 18 y 20 familias de alacranes, 159 géneros y 1270 especies (Fet, y cols., 2000). La familia Buthidae reúne 50 géneros, entre los cuales han sido encontrados los más peligrosos, tanto en el Viejo como en el Nuevo Mundo. El género *Centruroides*, perteneciente a esta familia, tuvo una excelente adaptación. Algunas de sus especies invadieron el

continente sudamericano, hasta la región chilena, siendo las únicas reportadas en zonas húmedas (Hoffman, 1932).

El género *Centruroides*

El género *Centruroides* fue descrito por primera vez en 1890 por G. Marx. La taxonomía de muchas especies de *Centruroides* es confusa y ha estado basada principalmente en caracteres morfológicos como la coloración y la morfoescultura. Existe una sola clave de identificación reconocida (Stahnke y Calos, 1977), la cuál está fuera de tiempo para las investigaciones que se realizan actualmente.

Centruroides (Marx, 1890), es uno de los géneros más diversos, con 41 especies y 24 subespecies (Fet y Lowe, 2000) dentro de la familia Buthidae (precedido solo por Tityus C. L. Koch con 46 especies y 22 subespecies). El género *Centruroides* solo se encuentra en el nuevo mundo y es el único género de la familia Buthidae encontrado en Norte América (Fet y cols., 2000). Son abundantes en diversos habitats naturales que van desde el bosque tropical hasta los desiertos, *Centruroides* es especialmente diverso en México (Lourenco y Sissom, 2000) y el Caribe (Armas, 1988), pero también se encuentra en Centro y Sudamérica (Sissom y Lourenco, 1987). Unas pocas especies dentro del género son tóxicas, y potencialmente letales para los humanos (Fet y cols., 2000).

Importancia médica de los alacranes

El alacranismo es un problema de salud pública, originado por la picadura de alacranes, que afecta grandes núcleos de población, tanto en el medio rural como en el urbano. En México hay 16 estados con especies peligrosas y se atienden alrededor de 50 mil personas al año afectadas por el alacranismo y la incidencia de su piquete. Gracias al uso del antiveneno, el número de muertes en la República ha disminuido considerablemente, de 700 que había en los años 70´s y 80´s, a 50 en el 2004 en estimaciones realizadas por la dirección general de epidemias de la Secretaría de Salud (SSA).

De más de 200 especies de alacranes reportadas en México, menos de una decena causan problemas de salud en el humano y todas pertenecen al género *Centruroides*. Las toxinas, son moléculas especie específicas, es decir, todos los alacranes producen componentes tóxicos en sus glándulas, pero solo algunas causan un efecto notable en el hombre. La mayoría de esas toxinas afectan a invertebrados artrópodos (crustáceos, chapulines, cucarachas, moscas, arañas etc.) con los que el alacrán generalmente se alimenta. Recientemente se han publicado algunas revisiones sobre las toxinas del veneno de alacranes, las cuales brindan una mayor información en este aspecto (Possani y cols., 2000; Rodríguez de la Vega y Possani, 2004). Los géneros de *Centruroides* y *Tityus* incluyen a los alacranes más dañinos para el hombre, su picadura puede provocar edemas locales, fiebre, naúseas ocasionales, vómito y una sensación extraña en la lengua que puede indicar respuesta alérgica. En la mayoría de los casos los síntomas pueden durar hasta 24 hrs (Maraboto, 1994). Los venenos en especies muy relacionadas difieren grandemente en toxicidad. Sin embargo, los estudios de Northey (1962), revelan que los venenos de diversas especies de la familia Buthidae y Vaejovidae poseen componentes antigénicos en común.

Debido al impacto en la salud de los humanos, los alacranes del género *Centruroides* son un grupo de alacranes que reciben una gran atención. Laurenco y Sissom, revisaron la diversidad de alacranes en México, enfatizando la necesidad de más investigación y la incorporación de nuevas técnicas (incluyendo técnicas de DNA) para entender la taxonomía compleja, origen y distribución de los *Centruroides* mexicanos (Lourenco y Sissom, 2000).

Análisis filogenéticos

Desde la epoca de Charles Darwin, uno de los sueños de los biólogos ha sido el de reconstruir la historia evolutiva de todos los organismos sobre la tierra y representarla como un árbol filogenético (Haeckel, 1866). Los árboles filogenéticos nos proveen de un registro indirecto de los eventos de especiación que se han dado a lo largo del tiempo (Barraclough y Nee, 2001).

En la actualidad existe un gran número de métodos de reconstrucción filogenética que están basados en distintos modelos evolutivos, por lo que potencialmente podríamos obtener más de un árbol filogenético para el grupo que se este estudiando. Es por esto que es necesario realizar

análisis estadísticos que nos permitan seleccionar el árbol con el mejor valor de soporte en sus ramas.

Cada método filogenético tiene sus ventajas y desventajas. Usando estos en paralelo nos ayudarán a descubrir que factores evolutivos están trabajando en los datos que se estén utilizando. Mencionaremos los aspectos más importantes de los principales métodos para la reconstrucción de árboles, y nos referiremos a la literatura para mayores detalles (Hillis, Moritz y Mable, 1996).

Maxima Parsimonia (MP)

Originalmente fué desarrollado para el análisis de caracteres morfológicos (Hennig, 1966), pero ha sido adaptado para funcionar también con datos moleculares (Fitch, 1971; Hartigan, 1973; Swofford, 1998). El método se basa en el supuesto de que la hipótesis más simple es preferible que las más complicadas, de esta manera selecciona el árbol más corto que requiera el mínimo número de pasos o cambios para explicar los datos. Cuando se comparan secuencias, el método solo utiliza los sitios variables los demás son eliminados.

El método de MP quizás de resultados más reales que otros métodos ya que esta libre de los supuestos evolutivos que son requeridos para otros (Miyamoto y Cracraft, 1991). Ha sido estudiado de manera extensiva matemáticamente, y algunas implementaciones poderosas de software están accesibles. Es un método sensible para medir el grado de heterogeneidad entre los linajes (conocido como atracción de ramas largas), y como otros puede asignar valores de bootstrap para mostrar la confiabilidad del árbol producido.

Neighbor Joining (NJ)

Este método de matriz de distancia desarrollado por Saitou y Nei en 1987, construye los árboles filogenéticos con los valores de las distancias evolutivas calculadas para cada par de taxas en la muestra.

La lógica detrás de todos los métodos de matriz de distancia es que si un par de secuencias de DNA difieren en un 10 % en los sitios de nucleótidos, estan más relacionadas entre sí que un par que difiere el 30%. Conforme más tiempo pase entre dos secuencias que divirgieron de un ancestro en común mayor será la diferencia entre ellas.

La reconstrucción de los árboles con este método, inicia con un árbol sin raíz en forma de estrella, es decir todos los taxas conectados en el centro. Utilizando las distancias calculadas se unen los taxas con las distancias más cortas y los siguientes hasta que se unen todos los taxas. La confiabilidad del árbol producido, está dado por los valores de bootstrap calculados.

Máxima Verosimilitud (ML)

El método de ML (por sus iniciales en inglés "Maximum Likelihood") para las inferencias filogenéticas, fué usado por primera vez en 1967 para datos de frecuencia genética (Cavalli-Sforza y Edwards, 1967). Es un método estadístico que da como resultado el o los árboles que con una mayor probabilidad se ajusten a los datos del estudio (Nei y Kumar, 2000). Cuando se utiliza el método de ML es necesario partir de un modelo evolutivo que describa tanto el rango como la probabilidad de que ocurra un tipo determinado de sustitución de nucleótidos. Si el modelo seleccionado se acopla bien a los datos del árbol se considera correcto. El programa ModelTest (Posada y Crandall, 1998) puede ayudar a seleccionar el modelo de evolución de DNA que mejor se ajuste a los datos comparando valores de likelihood. En contraste con otros métodos, ML hace uso de toda la información disponible (evidencia total).

Análisis Bayesianos (AB)

La estadística bayesiana está muy relacionada con los métodos de Máxima Verosimilitud (ML). La inferencia bayesiana de filogenia está basada en que la hipótesis óptima es la que maximiza la probabilidad posterior. La probabilidad posterior de una hipótesis es proporcional a la verosimilitud multiplicada por la probabilidad anterior de esa hipótesis. Las probabilidades previas llevan a los científicos a tener hipótesis sesgadas antes del análisis de los datos. En diversas aplicaciones los investigadores especifican las distribuciones de probabilidad anterior que ellos creen que son mayormente no-informativas, tal-que las mayores diferencias en la probabilidad posterior de las hipótesis son atribuibles a diferencias en la verosimilitud y provee medidas de soporte más rápidas que el bootstrap de máxima verosimilitud. Además los métodos bayesianos permiten implementar modelos complejos de evolución de secuencia como, la estimación de tiempos de divergencia, encontrar residuos importantes para la selección natural, y detectar puntos de recombinación. En los análisis bayesianos el resultado final no depende de un

valor específico, sino que considera los valores de todos los parámetros. Para los análisis bayesianos es necesario especificar la distribución anterior y el modelo evolutivo a utilizar, por ejemplo, el de dos parámetros de Kimura. Después de integrar el producto de estos valores sobre todos los parámetros posibles, se determina la probabilidad posterior de cada árbol. Las funciones de verosimilitud para modelos filogenéticos son muy complejas para integrarlas analíticamente por lo que las inferencias bayesianas recaen en el algoritmo de MCMC (de sus iniciales en inglés "Markov Chain Monte Carlo") que permite aproximar las distribuciones de probabilidad en una amplia variedad de contextos (Holder y Lewis, 2003).

Genómica y marcadores moleculares

El estudio de la evolución de los organismos se ha visto favorecido por los adelantos en cuanto a técnicas moleculares. La evolución a nivel molecular ocurre mediante la sustitución de un nucleótido o de un aminoácido por otro, el número de diferencias existentes en la secuencia de un ácido nucleico o de una proteína equivalentes en dos especies, revela en cierto modo, el tiempo transcurrido desde su ancestro común (Fu y Li, 1997). Los estudios evolutivos a nivel molecular presentan dos ventajas notables respecto a la anatomía comparada y a otras disciplinas clásicas. La primera consiste en que la información puede cuantificarse con mayor facilidad y la segunda en que es posible comparar organismos muy alejados evolutivamente o diferenciar aquellos que son muy similares morfológicamente (Fu y Li, 1997).

El uso de marcadores moleculares mitocondriales en estudios filogenéticos se vió incrementado a principios de los 90s por diversas razones. En primer lugar la gran cantidad de mitocondrias por célula sobre todo en tejido muscular favorece la obtención de DNA mitocondrial (DNAmt). Por otro lado el genoma es relativamente pequeño, de alrededor de 16 kb, lo que permite un fácil manejo y análisis. (Avise, 1994).

El DNAmt es una molécula circular de doble cadena, la cual generalmente carece de intrones y es heredada vía materna manteniendo una baja recombinación. En la mayoría de los organismos esta compuesta por 37 genes: 13 para proteínas, 2 para RNA ribosomales (RNAr) y 22 para RNAs de transferencia (RNAt) (Moritz y cols., 1987).

Los genes mitocondriales más utilizados en análisis filogenéticos son: 16S, 12S, ND4, COI y COII, aunque actualmente es posible realizar estudios tomando en cuenta el genoma mitocondrial completo de diversos organismos, principalmente en vertebrados y artrópodos.

La influencia de los datos moleculares en la sistemática empieza a jugar un papel importante en la búsqueda u observación de la historia evolutiva de los alacranes. El gene 16S ribosomal ha sido utilizado antes en alacranes (Fet y cols., 1999), y es ampliamente utilizado en estudios moleculares de la evolución de algunos artrópodos (Simon y cols., 1994).

ANTECEDENTES

Relaciones filogenéticas dentro del subphylum Chelicerata, clase Arachnida

El subphylum Chelicerata es uno de los grupos más controversiales, ha sido discutido durante más de 100 años, debido a su importancia tanto en la clasificación de Artropoda, así como en las relaciones filogenéticas de los ordenes que lo conforman la clase Arachnida. Haciendo una recapitulación de los trabajos realizados en este sentido, mencionaré los que considero más importantes.

La primera clasificación de los Arachnida, fué realizada por Thorell en 1877. En su trabajo toma en cuenta la complejidad morfológica y el grado de especialización de los distintos ordenes, nombrandolos como taxas "mayores" y "menores". Presenta a los Acaros como grupo basal y a los alacranes como un grupo derivado. Es el primero en incluir dentro de Uropygidos a Schizomida y Thelyphonida.

Lankester en 1881, incluye a Xiphosura dentro de Chelicerata debido a la similitud entre las agallas de libro en *Limulus* y los pulmones de libro en los alacranes. Divide a los Arachnida en Aerobranchia y Lipobranchia.

En 1893 Pocock, en total desacuerdo con Thorell sobre la posición de los alacranes (Ctenophora), presenta a estos en la base del árbol, separado de los demás Arachnida (Lipoctena). Pocock se basa en la diferencia del número de apéndices abdominales en embriones, que en los alacranes es de 6 y no más de 4 en los arácnidos restantes. Mantiene intacto el grupo Lipobranchia de Lankester, pero modifica Aerobranchia separandolo en Ctenophora (Scorpiones) y Caulogastra (Aranae, Amblypygi y Uropygi).

Snodgrass (1938), hizo la primera distinción básica entre Chelicerata y Mandibulata. Además estableció el esquema general de Chelicerata (Pycnogonida + Xiphosura + Arachnida) en base a caracteres firmes, el cual se ha mantenido hasta nuestros días.

Stormer en 1944, por otro lado excluyó a los Pycnogonidos del grupo Chelicerata e incluso de los trilobites no quelicerados.

Petrunkevitch (1955), presentó nuevos grupos. El primero llamado Labellata (Aranae + Amblypygi), esta basado en el parecido que encontró de la estructura circum-oral (boca

anteroventral entre 2 labios). Otro grupo, denominado Caulogastra (descrito anteriormente por Pocock) en donde incluyó además a Palpigradi, Solifugos y Ricinulei, y por último el grupo Latigastra compuesto por Scorpiones, Pseudoscorpiones, Opiliones y Acari el cual está basado en la unión entre prosoma y opistosoma.

En desacuerdo con Petrunkevich, Sharov en 1966, acertó al decir que los Scorpiones tienen un origen diferente de los demás arácnidos, esto aunque parece apoyar la posición basal de Scorpiones propuesta por Pocock, no es así ya que considera a los alacranes y arañas monofiléticos por la presencia de pedipalpos.

Savory 1971 niega la monofilia de Arachnida. Considera a los Cyphophthalmi como intermediario de Acari y Opiliones, estos junto con Ricinulei forman el grupo hermano de Labellata-Caulogastra. Y formando un nuevo clado presenta a los Scorpionomorpha (Solifugos + (Scorpiones + Pseudoscorpiones).

En un estudio exhaustivo de los Chelicerata, Firstman 1973 incluye a los grupos Pycnogonida, Palpigradi y Ricinulei, basandose en la variación del sistema arterial y su relación con el endosternito. Mantiene la posición basal de los alacranes. Propone a los Amblypygi como grupo hermano de los Aranae (Petrunkevitch en 1955, lo nombro Labellata) en base al número y posición del suspensor en el endosternito. Por último ubica a los Pycnogonida como el grupo hermano de los Chelicerata restantes, ya que considera que el septum vascular de los Pycnogonida es homólogo al endosternito de Xiphosura y Chelicerata.

Poco después, Yoshikura (1975) examina caracteres embriológicos, mantiene el grupo Labellata-Caulogasta. En contradicción con su dendograma, mantiene que los Uropygi y Amblipygi estan más cercanos. Los Scorpiones y Pseudoscorpiones aparecen como el grupo hermano de este clado. Los taxas restantes forman un grupo muy similar al propuesto por Pocock.

Weygoldt y Paulus en 1979, hacen una recopilación de la información de caracteres generada en los cien años previos añadiendo la suya propia. Ellos realizan el primer análisis cladista, produciendo un esquema de las relaciones entre Chelicerata basado en sinapomorfías. Pycnogonida fué incluido en la base como *incertae sedis*. Los Xiphosura aparecen como el taxon hermano de Arachnida. El esquema es muy parecido al de Pocock. La división básica entre Scorpiones (Ctenophora) y de Lipoctena que incluye a Caulogastra y Apulmonata (Solifugae, Pseudoscorpiones, Acari, Ricinulei y Palpigradi) esta bien sustentada.

En su análisis cladista de los artrópodos Weygoldt (1986), presenta claramente a los Pycnogonida como el grupo hermano de los euchelicerata (Xiphosura + Arachnida).

Através de la inclusión de datos morfológicos funcionales Shultz (1990) hizo resurgir el grupo de pedipalpi (Amblypygi + Uropygi) y como otros estudios presentó una combinación de grupos elaborados previamente. Su esquema básico incluye Caulogastra de Pocock y Scorpionomorpha de Savory, pero con Pseudoscorpiones formando un clado con Solifugos. La principal diferencia entre Shultz y Savory (además de la metodología) es la división de los Opilionoidea. Shultz incluye a Acari + Ricinulei con Caulogastra y a Opiliones con Scorpionomorpha.

En Wheeler y Hayashi 1998, la división convencional de Chelicerata (Pycnogonida + (Xiphosura + Arachnida)) esta bien sustentada tanto por datos morfológicos como moleculares. La principales discrepancias se presentan en los clados formados dentro de Arachnida, uno de los más sobresalientes es la posición de los alacranes, el cual aparece como un grupo derivado (Opiliones + Scorpiones + (Solifugids + Pseudoscorpiones)) casi idéntico al propuesto por Petrunkevitch (Latigastra pero excluyendo a Acari). Este arreglo coincide con el propuesto por Shultz pero varía con respecto al de Weygoldt y Paulus al no considerar a Scorpiones como un grupo basal. Los datos no apoyan la formación del clado Acari + Opiliones propuesto en trabajos previos, ni tampoco el grupo pedipalpi que resurgió en el trabajo de Shultz.

Estos tres últimos trabajos cladistas (Weygoldt y Paulus 1979; Shultz, 1990; Wheeler y Hayashi, 1998) muestran algunos clados constantes como el de Caulogastra (Aranae + Amblypygi + Uropygi) y el de Scorpionomorpha (Scorpiones + Pseudoscorpiones + Solifugae + Opiliones) los cuales estan respaldados por diversos caracteres. Algunos caracteres morfológicos como los de las piezas bucales sugieren que tanto los Opiliones como los Palpigradi son arácnidos basales. Los ácaros en general son considerados un grupo monofilético, frecuentemente aparecen como el grupo hermano de los Ricinulei, aunque el parentesco no es claro.

La posición de Scorpiones dentro de la filogenia de Chelicerata es una de las más polémicas ya que con ella se pone en duda la monofilia de Arachnida. Algunos autores (Shultz, 1990 y Wheeler y Hayashi, 1998) coinciden en considerar a la clase Arachnida monofilética, ya que presentan a los alacranes como un grupo derivado. Otros autores (Weygoldt y Paulus, 1979; Kjellesvig-Waering, 1986; Dunlop y Webster, 1999) que incluyen en el análisis a Eurypterida (organismos extintos) forman un clado basal con los alacranes apareciendo como el grupo hermano de los demás arácnidos (Lipoctena).

Como hemos podido observar en los distintos dendogramas realizados, las posiciones más inestables son: si Pycnogonida y Scorpiones son grupos basales o no y las relaciones internas principalmente de Opiliones, Solifugos y Pseudoscorpiones. También han sido cuestionadas la monofília de Pedipalpi, la unión de Aranae y Palpigradi y la tendencia de agrandar el grupo Caulogastra de Pocock. Es necesario aumentar la información y análisis evolutivos sobre estos taxa para esclarecer algunas de estas controversias.

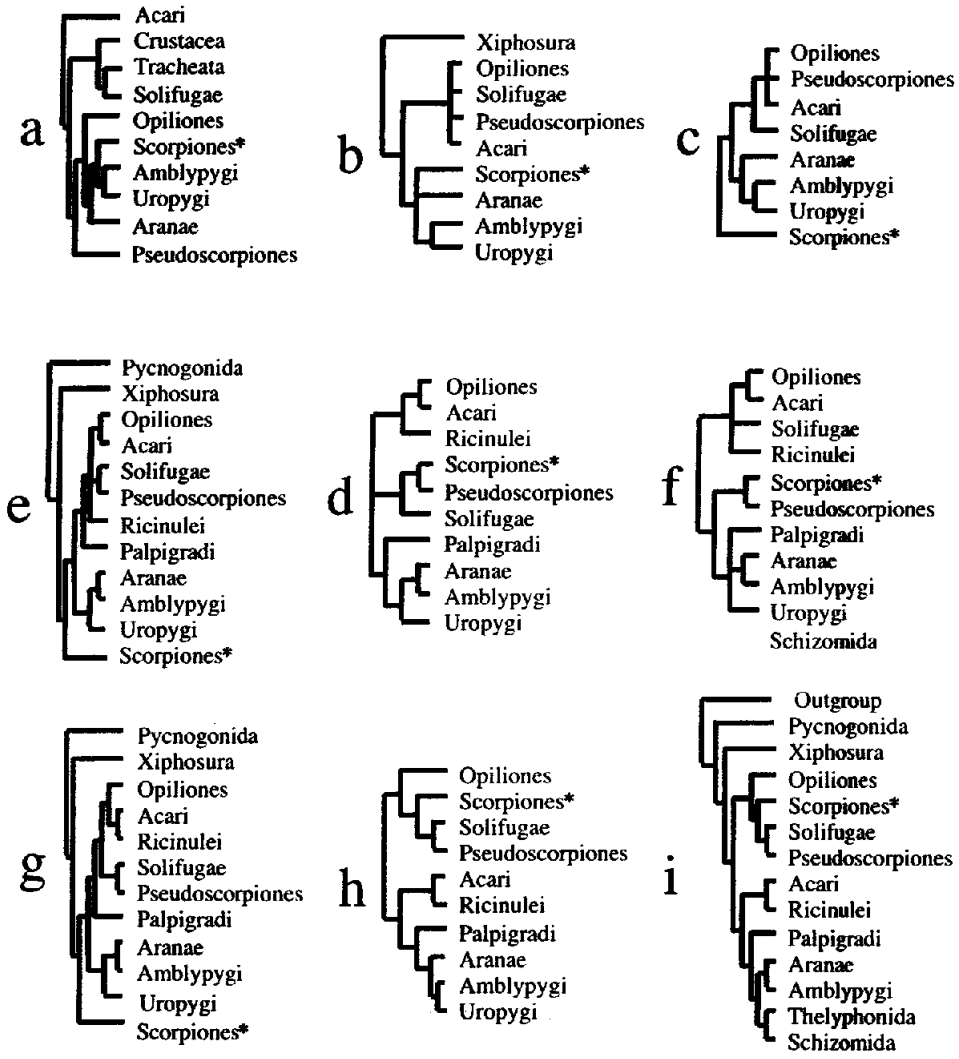

Figura 3 Arboles filogenéticos propuestos para el grupo Chelicerata. a) Thorell 1877, b) Lankester 1881, c) Pocock 1893, d) Savory 1971, e) Firstman 1973, f) Yoshikura 1975, g) Weygoldt y Paulus 1979, h) Shultz 1990 y i)Wheeler y Hayashi 1998.

Secuencias mitocondriales en Chelicerata

El genoma mitocondrial ha servido como sistema modelo en estudios genómicos, convirtiendose en una poderosa herramienta para la inferencia de las relaciones evolutivas entre distintos linajes.

En 800 millones de años de evolución, el contenido genético mitocondrial de los metazoa, ha permanecido prácticamente sin cambios, sin embargo los procesos que se han llevado a cabo nos permiten trazar la historia evolutiva de estos organismos. Estos procesos incluyen; rearreglos en el orden genético, cambios en la composición de las bases, el surgimiento de la asimetría composicional entre las dos cadenas de DNA, variaciones en el código genético, evolución en el uso de codones, tasas de sustitución nucleotídica linaje-específico y patrones evolutivos en las regiones de control (Saccone y cols., 2002).

Los artrópodos son los metazoa más abundantes, la incorporación de información molecular ha permitido determinar las relaciones filogenéticas entre los principales grupos vivientes que lo conforman (atelocerados, custáceos y quelicerados). Con la comparación de rearreglos genéticos mitocondriales es posible resolver relaciones filogenéticas entre linajes antíguos (Boore y cols., 1995).

Dentro de los artrópodos se encuentra el subphylum Chelicerata, uno de los grupos más diversos y antiguos así como controversiales en sus relaciones filogenéticas. Solo 18 secuencias mitocondriales han sido reportadas hasta el momento, incluyendo la obtenida en este trabajo (sitio web http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?CMD=search&DB=Genome).

Una de estas secuencias es del xiphosuro *Limulus polyphemus*, el cual debido a la condición basal del grupo al que pertenece, resulta ser muy útil en estudios filogenéticos y evolutivos (Lavrov y cols., 2000). Dentro de Arachnida el grupo con más secuencias es el de Acari (Shao y cols., 2005), estas secuencias han permitido determinar que los marcadores mitocondriales son más variables que los nucleares mediante patrones de polimorfismo intraespecífico (Navajas y cols., 1998). El orden genético mitocondrial de algunos Acari no corresponde al observado en *Limulus* (Black y Roehrdanz, 1998). Hasta el momento se han reportado 3 secuencias mitocondriales de arañas, la comparación de estas con otros Chelicerata han mostrado variaciones en el arreglo genómico, particularmente en la posición de los genes de tRNA. Lo más sobresaliente es que las estructuras inferidas de los tRNAs carecen de uno de los brazos y aparecen truncados en sus

extremos, además de la edición en los RNA. Todas estas son características que no comparten con los ácaros. Debido a que la modificación en la estructura secundaria de los tRNAs son eventos evolutivos poco frecuentes, es posible utilizarlos como caracteres filogenético para determinar linajes, aunque para esto es necesaria la secuenciación de otras mitocondrias dentro de Chelicerata (Masta y Boore, 2004).

Relaciones filogenéticas dentro del género *Centruroides*

Aunque Buthidae es la familia de alacranes más diversa y de mayor importancia médica (Fet y Lowe, 2000) pocos trabajos se han realizado en torno a su filogénia. La combinación de datos morfológicos y moleculares serían muy útiles en el establecimiento de la historia evolutiva de estos organismos (Gantenbein y cols., 1999; 2000). Los marcadores moleculares han ayudado a definir especies aisladas, donde ningún concepto de especie puede ser aplicado. Esto ha sido utilizado con 17 géneros de la familia Buthidae (Fet y cols., 2003) en el género *Buthus* Leach 1815 (Gantenbein y cols., 1999) y con 4 especies del género *Mesobuthus* Vachon 1950 (Gantenbein y cols., 2003).

Dentro del género *Centruroides* Marx (1890), la taxonomía y clasificación de algunas especies es confusa y su sistemática ha estado basada en características morfológicas y de coloración. Existen algunas claves de identificación de las especies de este género (Stahnke y Calos, 1977), pero pocos trabajos recientes se refieren a su taxonomía o filogenia, por esto es necesaria una revisión más detallada del grupo. En Norteamérica los buthidos estan representados por el género *Centruroides* del cual por lo menos 6 especies se consideran médicamente importantes (Keegan, 1980). Francke y Jones (1982), hacen un análisis completo de la historia de vida de *C. gracilis* lo que permite entender más sobre la variabilidad ontogenética y biología general de los alacranes. Sissom en 1987, reevaluó la posición taxonómica de 6 especies sudamericanas de *Centruroides* haciendo incapié en la descripción de *C. margaritatus* una de las especies más ampliamente distribuidas dentro del género. Los trabajos moleculares que incluyen especies del género *Centruroides* analizan la monofilia de las subespecies que componen a *C. limpidus* y *C. infamatus* localizados en la parte central de México (Towler y cols., 2001).

Hoffmann (1932) realizó una de las clasificaciones más importantes dentro del género *Centruroides* con especies mexicanas basandose en caracteres de coloración y morfológicos. En 1938 Hoffmann tomó en cuenta otras características, como la distribución geográfica y la toxicidad del veneno. En este sentido el reconoció las relaciones entre algunas especies independientemente de su morfología. El consideró la distribución y la toxicidad del veneno como una característica que podría agrupar a las distintas especies dentro del género.

Hoffmann hace hincapié en considerar que las especies tóxicas para los humanos están localizadas en el área que originalmente ocupaba la península meridiana del antiguo continente Norte americano, antes de la unión con las tierras del sur de los Neotropico.

La clasificación más reciente es una agrupación convencional también del género *Centruroides* presentando especies mexicanas propuesta por González (2001). En su estudio se describen cuatro grupos denominados: Gracilis, Bertholdii, Thorellii y el grupo Rayado.

HIPÓTESIS

Los alacranes son muy parecidos en sus rasgos generales, esto ha obligado a buscar características más finas para poder distinguirlos. Con el desarrollo de técnicas moleculares es posible evaluar las relaciones evolutivas que mantienen los alacranes con respecto a los demás Chelicerata así como de la filogenia en particular de las especies del género *Centruroides*.

En su evolución, los alacranes del continente Americano migraron hacia el sur a causa de una disminución en la temperatura. La radiación de los alacranes se llevó a cabo principalmente en México, ya que fué hasta el Mesozoico que Norte América y Sudamérica se unieron por el Istmo de Panamá.

Los alacranes de la familia Buthidae, se encuentran alrededor del mundo entre los 52^0 latitud Norte y 42^0 latitud Sur. Esta es la familia más primitiva y antigua, originada en Laurasia y dispersada hacia Gondwana. El género *Centruroides* sólo se localiza en el nuevo mundo por lo que es posible pensar que el origen de este género se llevo a cabo en Norte América y con el tiempo se fueron dispersando hacia Sudamérica.

OBJETIVOS

General:

Proponer mediante herramientas de reconstrucción filogenética, las relaciones evolutivas existentes entre los alacranes a distintos niveles jerárquicos.

Específicos:

1. Obtener la secuencia del genoma mitocondrial de *C. limpidus*, con el fin de estudiar la posición filogenética de los alacranes dentro de Chelicerata.
2. Evaluar los marcadores moleculares mitocondriales adecuados para el análisis de las relaciones evolutivas del género *Centruroides*.
3. Realizar un análisis filogenético a partir de caracteres morfológicos y morfométicos del género *Centruroides*.
4. Utilizar marcadores moleculares mitocondriales para la determinación de las relaciones filogenéticas (COI, COII) en el género *Centruroides*.
5. Comparar las filogenias obtenidas de los diferentes datos y hacer una contribución a la taxonomía y clasificación del género *Centruroides*.

CAPITULO II

Resumen

En este trabajo se hace un análisis de la secuencia del genoma mitocondrial de *Centruroides limpidus*. La secuencia fué obtenida mediante técnicas comunes en biología molecular, las cuales incluyen, reacciones de PCR, fragmentación de DNA, clonación, y secuenciación entre otras. Durante la escritura del artículo otra secuencia de alacrán fué liberada en la base de datos. Esto permitió hacer comparaciones en cuanto al contenido y organización de los genes mitocondriales en alacranes, así como determinar aquellas características comunes entre ellos.

Los principales resultados obtenidos muestran que la organización genética es muy parecida a la de *Limulus polyphemus* (especie basal dentro de Chelicerata) a excepción de que *C. limpidus* carece del gene para el RNA de transferencia de Aspártico y muestra una inversión en la posición de los genes para los RNA de transferencia de Glutamina e Isoleucina. Este último rearreglo en *C. limpidus* también lo presenta *Mesobuthus gibbosus*, el otro alacrán secuenciado. Otro dato interesante es que algunos de los genes que se codifican carecen del codon de terminación convencional, mediante los experimentos realizados se observó que al menos en dos productos se efectuaba la poliadenilación postranscripcional en el extremo 3', lo que sugiere que no es necesario el codon canónico de término para realizar una traducción adecuada. La estructura secundaria obtenida de los RNA de transferencia no muestran la estructura tradicional de trébol, esta característica la comparte con *M. gibossus* y con las arañas. La comparación de la secuencia mitocondrial de los dos alacranes nos muestra que *M. gibossus* es mayor, esta diferencia de tamaño se encuentra en la región no codificante. El contenido de AT es muy parecido entre los dos genomas. La identidad entre las proteínas que codifican es mayor de 49% y los genes de RNA ribosomales mantienen una identidad de 65%.

Por último, en las inferencias filogenéticas encontramos que los alacranes forman un clado que aparece como grupo hermano de Aranae y claramente separado de Acari. Sin embargo, la falta de información de los demás ordenes dentro de Arachnida, impiden establecer a ciencia cierta la posición filogenética de los alacranes, aunque los datos sugieren que los alacranes no son el grupo basal propuesto por Weygoldt y Paulus en 1979.

The mitochondrial genome sequence of the scorpion *Centruroides limpidus* (Karsch 1879) (Chelicerata; Arachnida)

Sonia Dávila [a], Daniel Piñero [b], Patricia Bustos [a], Miguel A. Cevallos [a], Guillermo Dávila [a,*]

[a] *Programa de Genómica Evolutiva, Centro de Ciencias Genómicas, Apartado Postal 565-A, Cuernavaca, Morelos, México. Universidad Nacional Autónoma de México*

[b] *Departamento de Ecología Evolutiva, Instituto de Ecología, C.P. 04510, D. F., México. Universidad Nacional Autónoma de México*

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Abstract

The mitochondrial genome of the scorpion *Centruroides limpidus* (Chelicerata; Arachnida) has been completely sequenced and is 14,519 bp long. The genome contains 13 protein-encoding genes, two ribosomal RNA genes, 21 transfer RNA genes and a large non-coding region related to the control region. The overall A+T composition is the lowest among the complete mitochondrial sequences published within the Chelicerata subphylum. Gene order and gene content differ slightly from that of *Limulus polyphemus* (Chelicerata: Xiphosura): i.e. the lack of the *trnD* gene, and the translocation-inversion of the *trnI* gene. Preliminary phylogenetic analysis of some Chelicerata shows that scorpions (*C. limpidus* and *Mesobuthus gibbosus*) make a tight cluster with the spiders (Arachnida; Araneae). Our analysis does not support that Scorpiones order is the sister group to all Arachnida Class, since it is closer to Araneae than to Acari orders.

Keywords: mtDNA; phylogenetic inference; tRNA secondary structure; Arthropoda.

Abbreviations:

atp6 and *atp8*, genes for ATP synthase subunits 6 and 8 (ATP6 and ATP8, protein products); *cox1-3*, genes for cytochrome *c* oxidase subunits I-III (COI and COII, protein products); *cob*, gene for cytochrome *b* (COB protein product); *nad1-6* and *nad4L*, genes for NADH dehydrogenase subunits 1-6 and 4L (NAD1-6 and NAD4L, protein products); *rrnS* and *rrnL*, genes for the small and large subunits of ribosomal RNA (s-rRNA and l-rRNA products); *trnX*, transfer RNA genes with corresponding amino acids denoted by one-letter code; $tRNA^{Xxx}$, transfer RNA with corresponding amino acids denoted by three-letters code; aa, amino acid(s); bp, base pair; mtDNA, mitochondria(l) DNA; PCR, Polymerase Chain Reaction; ORF, Open Reading Frame; MP, Maximum Parsimony; cDNA, DNA complementary to RNA. Nucleotide symbol combination V=A/C/G; N= A/T/G/C/.

* Corresponding author. Tel.: +52 777 3133881; fax: +52 777 3175581. E-mail address: davila@ccg.unam.mx (G. Dávila).

1. Introduction

Centruroides limpidus is one of the 1,259 living scorpion species described (Fet, *et al.*, 2000). The scorpions of *Centruroides* genus are known as the most abundant and dangerous species for humans. They account, only in Mexico, at least for 200,000 sting accidents per year (Dehesa-Davila, 1989). *C. limpidus* is found in arid and semiarid habitats of Central and South parts of Mexico.

The order Scorpiones is a highly diverse group of organisms that embraces 155 genera in 16 families with a controversial phylogenetic position within the Arachnida: some authors support that the scorpions are a sister taxon of the remaining arachnids (*i. e.* Weygoldt and Paulus, 1979), but others, based on molecular and/or morphological data sustain that the scorpions are close related to the Solifuges (sun spiders) and the Pseudoscorpiones (false scorpions) (Wheeler and Hayashi, 1998; Shultz, 1990).

In animals, mitochondrial DNA (mtDNA) is a single circular duplex molecule generally ranging in size from 15 to 17 kb. Despite their differences in size, almost all of them contain 13 protein-coding genes, 22 *trn* genes (transfer RNA genes), and two *rrn* genes (ribosomal RNA genes) (Boore, 1999). Metazoan mtDNA also contains a large non-coding region probably involved in the control of transcription and/or DNA replication (Wolstenholme, 1992). The mitochondrial genome has been extensively used to study the phylogenetic relationships at several taxonomic levels, mainly because it's maternal inheritance, the fast evolutionary rate compared to that of the nuclear DNA, and the lack of intermolecular genetic recombination. It has been shown that the order of mtDNA genes is generally conserved within the metazoa and that the gene rearrangements could be used to deduce deep-level phylogenetic relationships (Boore, *et al.*, 1995).

Until now, the mitochondrial DNA sequence of 605 metazoan species has been determined, however, only 90 of them belong to the most diverse phylum, the Artropoda. Nevertheless this kind of information is uneven: some Arthropoda classes are over represented while others are incomplete or absent.

In this study, we report the nucleotide sequence of *C. limpidus* mitochondrial genome. This genome and the *Mesobuthus gibbosus* mitochondrial genome recently deposited in GenBank are the first's representatives of the Scorpiones order. Our preliminary phylogenetic analysis shows that the scorpions clustered with the spiders (Arachnida; Araneae) leaving ticks and mites (Arachnida; Acari) as an external clade, not supporting Scorpiones order as a sister group to all the Arachnida.

2. Materials and methods

2.1 Sample and DNA extraction

Genomic DNA was extracted from one specimen of *Centruroides limpidus* collected in Cuernavaca Morelos, México, and identified using the keys to the species of Hoffman, (1932), Díaz Nájera (1966), Stahnke and Calos (1977) and Armas, *et al.* (1995). Total DNA was obtained from the complete organism homogenized with liquid nitrogen and then using DNeasy Plant Mini Kit (Quiagen), following the manufacturers protocol with the centrifugation at 4,000 rpm, equivalent to 1,500 *g*.

2.2 PCR and sequencing

The mitochondrial genome sequence was obtained from four overlapping PCR products. The largest amplification product had a 10,076 bp in size, and was obtained utilizing primers Cli06 and HPK16Sbb (Hwang, *et al.*, 2001). The 4,092 bp amplicon was obtained using primers HPK16Saa (Hwang, *et al.*, 2001) and Cli05 (Figure 1). These two products comprehend almost all the scorpion mitochondrial genome, but to complete it, two extra-set of PCR primers were designed to yield two amplification products overlapping with the first pair. One of these was obtained using primers Cli02 and Cli08 and yielded a PCR product of 964 bp. The primers Cli14 and Cli18 yielded an amplification product of 1,094 bp. All Cli primers were based on the sequence from *C. limpidus*. All PCR reactions were done with Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) using the conditions suggested by the manufacturer.

Reaction conditions were 1 cycle of 5 min at 94°C, 5 cycles of 30 s at 94°C, 45 s at 50°C, and 1 min per kb amplified at 68°C, 20 cycles of 30 s at 94°C, 45 s at 60°C, and 1 min per kb amplified at 68°C, and 1 cycle of prolonged elongation for 10 min at 72°C.

PCR products were loaded onto 1% TAE agarose gel with appropriate DNA size marker and bands were observed on a UV transilluminator to estimate their size and concentration. The PCR products were purified from the gel using the QIAquick ™ Gel Extraction Kit (Quiagen). The two larger amplification products were randomly shattered with a VixOne™ nebulizer (Westmed). Fragments between 1.5 and 2 kb were purified

from an agarose gel, enzymaticaly repaired and ligated into pZero vector (Invitrogen). The ligation products were transformed into *E. coli* TOP10' (Invitrogen) to create plasmid libraries, all using standard techniques (Sambrook and Russell, 2001). The sequencing reactions were made using the BigDye Terminator Cycle Sequencing Ready Reaction kit, and run in an AbiPrism 3700 DNA Sequencing System (Applied Biosystems). Three hundred and six clones were sequenced by both sides using universal primers, the final sequence was assembled using the public domain computer software CONSED (http://bozeman.mbt.washington.edu/phredphrapconsed.html; Ewing, *et al.*, 1998) with a confidence value of 0.83 X 10-Kb and a 10.5 X coverage in average. The DNA sequence was deposited in GENBANK under accession number AY803353.

2.3 Sequence analysis

The putative Open Reading Frames (ORFs) were identified by Gene Finder program available in NCBI using the mitochondrial genetic code. Similarity searches were done using BLAST (Altschul, *et al.*, 1990). Both programs are available at the NCBI web site (http://www.ncbi.nlm.nih.gov/gorf/gorf.html and http://www.ncbi.nlm.nih.gov/BLAST/, respectively). The boundaries of the genes were annotated minimizing the overlapping between genes. The initial start codon was selected as the first legitimate in-frame start codon (ATN, GTG, TTG, and GTT), not overlapping the ORF of the gene encoded previously in the same DNA strand. The stop codon was located and annotated as the first in-frame stop codon found. However if the stop codon was located within the ORF encoded in the same DNA strand, the last T or TA prior to the start codon of the next gene was designated as the termination codon. This decision was made taking into account that the polyadenylation process can reconstitute the stop codon (Ojala, *et al.*, 1981). In this paper we used the gene nomenclature proposed by Boore (1999), and by the commission on Plant Gene Nomenclature (1994).

Five *trn* genes were identified using the computer software tRNAscan-SE 1.21 (Lowe and Eddy, 1997, http://www.genetics.wustl.edu/eddy/tRNAscan-SE/), with the default parameters for mitochondrial DNA. The rest but two were identified with the search parameters described by Masta and Boore (2004), Source: Nematode mito, tRNA Cove cutoff score: 0.01. The last two *trn* genes were found manually as sequences with the required anticodon and a clover-like secondary structure. The boundaries of the *rrn* genes were identified by sequence similarity with other mitochondrial *rrn* genes of arthropods.

The nucleotide composition skewness was calculated for each DNA strand using the method of Perna and Kocher (1995), where AT skew = [A-T]/[A+T] and the GC skew= [G-C]/[G+C].

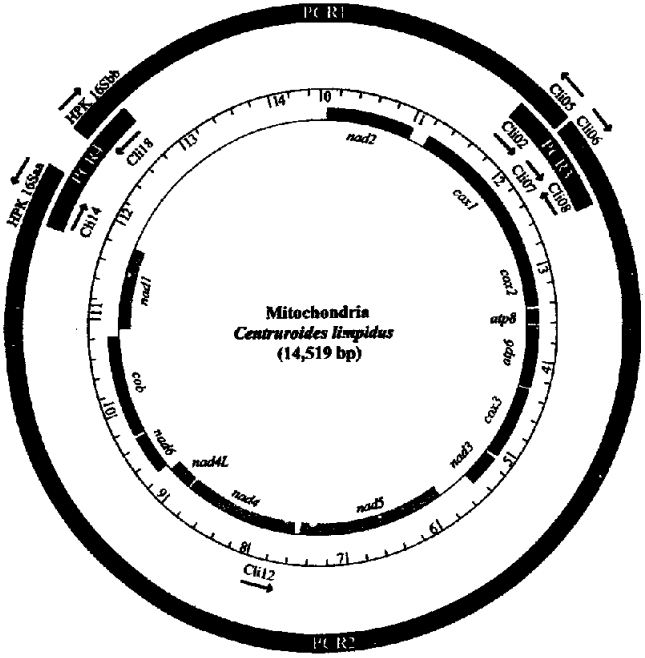
2.4 Phylogenetic analyses

Besides the sequence of the *C. limpidus* mitochondrial genome reported here, the data set for our phylogenetic analysis were obtained from GenBank and includes the mitochondrial genome sequences of representative species of the Chelicerata; Mediterranean checkered scorpion (*Mesobuthus gibbosus*, NC_006515); jumping spider (*Habronattus oregonensis*, NC_005942); spider (*Heptathela hangzhouensis*, NC_005924); hedgehog tick (*Ixodes hexagonus*, NC_002010); Chinese earth tiger (*Ornithoctonus huwena*, NC_005925); soft tick (*Ornithodoros moubata*, NC_004357); brown dog tick (*Rhipicephalus sanguineus*, NC_002074) and honeybee mite (*Varroa destructor*, NC_004454). The sequence of the Atlantic horseshoe crab *Limulus polyphemus* (NC_003057) mitochondrial genome was selected as an out-group.

The phylogenetic tree presented here was constructed as follows: The amino acid sequences of individual proteins were aligned using ClustalW (Chenna, *et al.*, 2003) with default penalties and manually edited. Different data sets were analyzed since some of the mitochondrial proteins are not useful for reconstructing the phylogenies because of their small size and high variation. The first data set consists of the 13 concatenated aligned proteins. In the second set ATP8 and NAD6 were eliminated from the concatenated alignment. The third set is similar to the previous one but without NAD2. In the fourth set ATP8, NAD6 and NAD4L were excluded from the alignment. In the fifth data set NAD2, ATP8, NAD6 and NAD4L, were not included in the analysis. The last data set consists of the concatenated alignment of the four most conserved proteins: COI, COII, NAD1 and COB.

An unrooted tree of each set of proteins was constructed using maximum parsimony (MP) analyses implemented in PAUP*4.0b10 (Swofford, 1998). Parsimony analyses were performed using 1000 bootstrap replicates with random addition of taxa and tree-bisection reconnection branch swapping (Felsenstein, 1985).

A



B

PCR	Primer	Sequence	Genome position	PCR product size	Overlaping
1	HPK 16San	5'ATGCTACCTTTGCACRGTCAAGATACYGCGGC	*rrnl*	4092bp	
1	Chi05	5'CATACCCAAAGARCCAAAAGG	*cox1*		
2	Chi06	5'GTKTGGGCTCATCATATG	*cox1*	1087bp	
2	HPK 16Sbb	5'CTTATCGAYAAAAAAGWTTGCGACCTCGATGTTG	*rrnl*		
3	Chi02	5'ATTAATATGCGGAGAAGTGG	*cox1*	964bp	PCR1'(316bp)
3	Chi08	5'AGGCATATAACTAGCTCCAG	*cox1*		PCR2'(609bp)
4	Chi14	5'TCTGAATATAGCCTAGCACC	*nad1*	1894bp	PCR2'(420bp)
4	Chi18	5'CTCGAGAAAAACACAAACTG	*rrnl*		PCR1'(362bp)
5	Chi07	5'GTATTGTTAGTTCTCGTGGG	*cox1*	~90bp	
6	Chi12	5'ATTAATCTCCTTTCTCTCTG	*nad4*	~160bp	

Figure 1. A) Mitochondrial gene map of *Centruroides limpidus* with the localization and names of the primers used for amplification. Arrows indicates the primer directions. Black thick lines represent the PCR amplification products. The genes encoded in the α-strand are shown in dark grey, and in light grey those encoded in β-strand. B) Table containing features and sequences of the primers used in this work.

All phylogenetically uninformative sites were ignored and gaps were considered as missing data.

The Tajima relative rate test (Tajima, 1993) was performed to evaluate the homogeneity of rate substitution within the Araneae group, implemented in the MEGA v. 2.1 program (Kumar, *et al.*, 1993).

2.5 Comparative table

A table comparing the Chelicerata mitochondrial genome properties was constructed using the available information deposited in GenBank that include the genomes already mentioned in the phylogenetic analysis

and the mtDNA sequences of: spider (*Heptathela hangzhouensis,* NC 005924); Ornate kangaroo tick (*Amblyomma triguttatum* NC 005963), softbacked tick (*Carios capensis* NC 005291), hardbacked tick (*Haemaphysalis flava* NC 005292), paralysis tick (*Ixodes holocyclus* NC 005293), taiga tick (*Ixodes persulcatus* NC 004370), common seabird tick (*Ixodes uriae* NC 006078), and soft tick (*Ornithodoros porcinus* NC 005820).

2.6 RNA isolation and 3'-end determination of cox1 and nad4 transcripts

To determine the 3'-ends of the *cox1* and *nad4* transcripts, total RNA was isolated from the first six tergites of the scorpion opisthosoma using the Total RNA isolation system (Promega). The full-length cDNAs were obtained following the protocol instructions for the SMART cDNA Kit (Clontech). To determine the 3'-ends of the *cox1* and *nad4* transcripts a PCR reaction was made, using as template 100 ng of total cDNA, the universal primer CDS III/3'[5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)$_{30}$VN -3'] which is common to all the 3'ends of cDNA, and primers Cli06 and Cli02 to amplify *cox1* and *nad4,* respectively. The amplification reactions were cycled at 1 cycle of 5 min at 94°C, 5 cycles of 30 s at 94°C, 45 s at 55°C, and 30 s at 68°C, 20 cycles of 30 s at 94°C, 45 s at 62°C, and 30 s per at 68°C, and 1 cycle of prolonged elongation for 10 min at 72°C. The PCR products were purified from the gel using the QIAquick ™ Gel Extraction Kit (Quiagen). The PCR products were cloned using with the TOPO TA Cloning Kit (Invitrogen) using the protocol suggested by the manufacturers. Recombinant plasmids containing the desired inserts were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit, and run in an AbiPrism 3700 DNA Sequencing System (Applied Biosystems).

3. Results and discussion

3.1 Genome content and gene order

The mitochondrial genome of the scorpion *C. limpidus* is 14,519 bp long and contains 13 protein-encoding genes, two *rrn* genes and a large non-coding sequence, the probable control region, located between *rrnS* (small mitochondrial rRNA subunit) and *trnQ*. Only 21 *trn* genes were identified instead of typical 22 found in the metazoan mitochondrial genomes (Boore, 1999). We were unable to detect the presence of *trnD,* but this is not an exception: *trnQ* has not been identified in the mitochondrial genome of the whitefly *Aleurodicus dugesii, trnS1* in the mitochondria of the aphid *Schizaphis graminum* (Thao, *et al.,* 2004) and *trnV* in the mitochondrial DNA of the freshwater crayfish *Cherax destructor* (Miller, *et al.,* 2004).

Table 1
Gene content and properties of the mitochondrial genome of *C. limpidus*

Gene	Position	Size	Strand	Start	Stop	3'spacer
trnM	1-60	60	α	-	-	0
nad2	61-1022	962	α	TTG	TA	0
trnW	1023-1084	62	α	-	-	-3
trnC	1082-1136	55	β	-	-	2
trnY	1139-1204	66	β	-	-	0
cox1	1205-2737	1533	α	TTG	TAG	3
cox2	2741-3413	693	α	ATG	T	0
trnK	3414-3477	64	α	-	-	0
atp8	3478-3633	156	α	GTG	TAG	-7
atp6	3627-4292	666	α	ATG	TAG	3
cox3	4296-5076	781	α	ATG	T	0
trnG	5077-5131	55	α	-	-	0
nad3	5132-5471	340	α	ATT	T	0
trnA	5472-5531	60	α	-	-	1
trnR	5533-5587	55	α	-	-	2
trnN	5590-5651	62	α	-	-	-14
trnS1(agc)	5638-5697	60	α	-	-	5
trnE	5703-5758	56	α	-	-	2
trnF	5761-5821	61	β	-	-	0
nad5	5822-7499	1687	β	ATG	T	3
trnH	7503-7558	56	β	-	-	0

nad4	7559-8879	1321	β	ATA	T	-4
nad4L	8876-9163	261	β	ATG	TAA	1
trnT	9165-9222	58	α	-	-	0
trnP	9223-9282	60	β	-	-	2
nad6	9285-9717	433	α	ATA	T	0
cob	9718-10816	1099	α	ATG	T	0
trnS2(uca)	10817-10879	63	α	-	-	15
nad1	10895-11812	918	β	ATT	TAA	0
trnL2(uua)	11813-11873	61	β	-	-	-2
trnL1(cua)	11872-11931	60	β	-	-	0
rnl	11932-13063	1132	β	-	-	0
trnV	13064-13123	60	β	-	-	0
rns	13124-13850	727	β	-	-	545
trnQ	14396-14456	61	α	-	-	2
trnI	14459-14519	61	α	-	-	0

The sequence of the *C. limpidus* mitochondrial genome was numbered beginning with the first nucleotide of the *trnM* gene. In the 3′-spacer column, negative numbers indicate that genes are overlapped.

The DNA strand carrying most of the genes, named α, contains 9 protein coding genes and 13 of the 21 *trn* genes (Table 1). In the other strand (β) are located the two *rrn* genes, 8 *trn* genes and 4 protein-encoding genes. The 36 genes present in the mitochondrial genome of *C. limpidus* are organized in 8 directons (group of genes encoded in the same strand without interruptions); from which two of them are monocistronic. We found five pairs of overlapping genes. The largest overlapped region (14 bp) is located between *trnN* and *trnS1* (Table 1).

The gene order found in the mitochondrial genome of *C. limpidus* differs from the gene arrangement present in the mitochondrial genome of the horseshoe crab *Limulus polyphemus* (Lavrov, *et al.*, 2000; Boore, *et al.*, 1995) by the absence of *trnD* and the translocation-inversion of *trnI* (Figure 2).

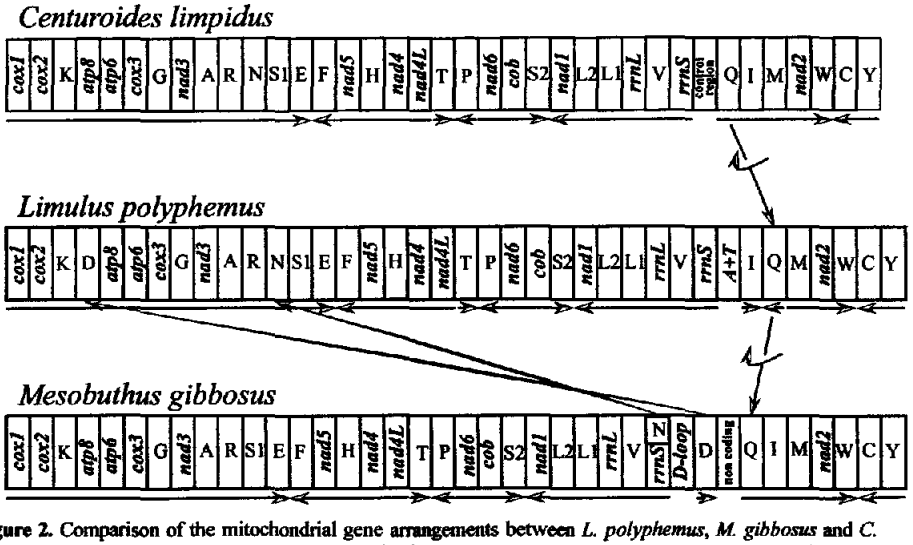


Figure 2. Comparison of the mitochondrial gene arrangements between *L. polyphemus*, *M. gibbosus* and *C. limpidus*. The genome maps were arbitrary linearized at the *cox1* gene. Genes are not to scale. The directions of transcription are indicated with arrows. And the arrangements are outlined with connecting arrows.

Table 2 Characteristics of the Chelicerata mitochondrial genomes

Species	Genome size	Protein genes	*rrn* genes	*trn* genes	Control region	Gene order	%AT
Centruroides limpidus	14519	10850	1859	1286	545	2	64.46
Mesobuthus gibbosus	15681	10763	1909	1324	1134	3	68.32
Limulus polyphemus	14985	11077	2095	1473	348	0	67.57
Haemaphysalis flava	14686	10817	1895	1342	310X2	3	76.91
Amblyomma triguttatum	14740	10876	1892	1383	307X2	6	78.35
Habronattus oregonensis	14381	10756	1709	1165	717	6	74.34
Varroa destructor	16477	10728	1875	1406	2174	7	80.02
Ornithoctonus huwena	13874	10733	1714	1240	396	5	69.79
Ornithodoros moubata	14398	10890	1898	1335	342	0	72.26
Ornithodoros porcinus	14378	10877	1898	1425	338	0	70.98
Ixodes persulcatus	14539	10888	1926	1398	352	0	77.33
Ixodes hexagonus	14539	10826	1992	1381	359	2	72.65
Ixodes uriae	15053	10837	1922	1371	476	0	74.78
Ixodes holocyclus	15007	10860	1930	1379	450	0	77.37
Rhipicephalus sanguineus	14710	10803	1877	1347	305	3	77.96
Carios capensis	14418	10873	1920	1342	342	0	73.54
Heptathela hangzhouensis	14215	10765	1817	1285	340	0	72.21

For each mtDNA, total lengths (in base pairs) of the genome, protein genes, *rrn* genes, *trn* genes and control region are shown. All genomes contain genes in both strands. Gene order is expressed by the minimum number of rearrangements to interconvert the gene map (protein coding genes, *rrn* genes and *trn* genes) to that of *Limulus polyphemus*. The AT content of each genome is also shown.

3.2 Base composition

The A+T content of the *C. limpidus* mitochondrial genome is 64.46% and represents the lowest A+T content found within Chelicerates (Table 2). The α strand has the following nucleotide composition: A= 25.9%; C= 13.2%; G= 22.4%; T= 38.6%, with a GC-skew= 0.26 and AT-skew= -0.19. The AT-skew is higher in *C. limpidus* than in all other published arthropods including *Locusta migratoria* (Flook, *et al.*, 1995) with an AT-skew of 0.18, reflecting their base-compositional differences. An asymmetry consistent with a mitochondrial replication-induced mutation bias (Francino and Ochman, 1997).

3.3 Gene initiation and termination

Of the 13 protein-encoding genes present in the mitochondrial genome of *C. limpidus*, 6 of them use ATG as start codon, while the rest use alternative start codons such as those found in other animal mitochondrial genomes. Alternative start codons TTG, ATT and ATA are used two times each, while GTG is utilized only once. Complete stop codons without overlap of the downstream gene are present in six genes (TAA and TAG, three times each). The *cox2*, *nad3* and *cob* genes probably also finish with a TAG codon, however, in these three genes the AG nucleotides of the stop codon imbricate with the 5' end of the *trnA* gene located downstream, and in consequence, they were annotated as finishing in the T to minimize overlapping. The stop codon of the other protein coding genes seems to be completed by cleavage of the transcript followed by polyadenylation (Ojala, *et al.*, 1981; Cattaneo, 1991) see Table 1. To test this assumption the 3'-ends of the *cox1* and *nad4* transcripts were identify as described in materials and methods. The *cox1* gene has a *bona fide* stop codon; meanwhile the *nad4* transcript supposedly requires polyadenylation to form the termination codon. The two transcripts were rescued by PCR from a cDNA pool obtained from RNA isolated from the scorpion opisthosoma. The sequence of the amplified products shows that both transcripts were polyadenylated, as observed in many other mitochondrial transcripts (*i. e.* Gagliardi *et al.*, 2004). The poly(A) tail, in the *cox1* transcript, was added just three nucleotide residues after the stop codon. In contrast, in the *nad4* mRNA the poly(A) was appended immediately after the last amino acid encoding triplet, suggesting that a canonical stop codon is not required for its adequate translation. Further experimentation is needed to support this observation.

3.4 Transfer RNAs

Only 21 *trn* genes were identified for the *C. limpidus* mtDNA, instead of the 22 *trn* genes usually found in metazoan genomes (Figure 3). The gene for *trnD* appears to be absent or its structure is so atypical that our criteria to find it failed. Five *trn* genes (*trnW, trnY, trnK, trnL1* and *trnL2*) were identified using the computer software as described in section *2.3*. The rest of *trn* genes were found modifying the search parameters as described by Masta and Boore (2004). In our first approach, *trnS1* and *trnI* genes were found overlapping the *rrnL* (large mitochondrial rRNA subunit) gene. Because of this, alternative positions were found manually as sequences with the adequate anticodon, identity, and the potentiality to form a clover-like secondary structure. The *trnN* and the *trnS1* genes imbricate 14 bp, nevertheless, their relative position is the same to that present in the genome of *Limulus polyphemus* (Lavrov, *et al.*, 2000). This high degree of gene overlapping is uncommon however a similar situation has been reported in the *trnY* and *trnC* genes of the spider *Habronattus oregonensis* (Masta and Boore, 2004). The size of the *trn* genes found in this genome ranged from 55 to 64 bp, a few base pairs shorter than the 66 bp found in the average *trn* gene of *C. limpidus*.

The *C. limpidus* mitochondrial tRNAs have some features that deviate them of the classical clover leaf structure: First, in fourteen tRNAs the TψC arm has been substituted by a loop of variable size (TV-replacement). Second, the putative secondary structure of $tRNA^{Ser1}$ has a D-replacement loop as described for metazoans (Garey and Wolstenholme, 1989); however this type of modification is present also in the predicted secondary structures of the $tRNA^{Arg}$, $tRNA^{Asn}$, and $tRNA^{Gln}$. Third, poorly paired aminoacyl acceptor stems were found in $tRNA^{Asn}$, $tRNA^{Ser1}$ and $tRNA^{Glu}$, an uncommon circumstance but present in several mitochondrial tRNAs of the spider *H. oregonensis* (Masta and Boore, 2004). The CCA signature present in the 3' end of the metazoan tRNAs is present in only one gene: *trnC*. In the rest of the tRNAs the CCA sequence needs to be added post-transcriptionally and after cleavage as demonstrated in the mitochondria of the land snail *Euhadra herklotsi* (Yokobori and Pääbo, 1995a). Fourth, inferred anti-codon stem of the tRNAs has the usual metazoan 5 bp except in *trnW*, *trnK*, *trnS*, *trnI*, *trnE* and *trnH* which contain a 4 bp stem. The nucleotide preceding the anticodon is U and before that a pyrimidine, except in *trnQ*. The anti-codon nucleotides for the corresponding *trn* genes are identical to those usually found in other mitochondrial genomes.

3.5 Ribosomal RNAs genes

As all other metazoan, the mitochondrial genome of *C. limpidus* contains the *rrnS* and *rrnL* subunits. Both genes are separated by *trnV* in an identical arrangement to *Limulus* and many other metazoans (Boore, 1999). The 5' and 3' ends of *rrnL*, and the 3' end of *rrnS* were determined by sequence comparison with other Arthropoda *rrn* genes, taking also into account the boundaries of the *trnV* and *trnS1* genes. However, the 5' end of *rrnS* was only inferred by sequence comparisons with the equivalent genes of other Arthopoda mitochondrial genomes.

Previously, the sequences of several subspecies of the *C. limpidus rrnL* gene were reported (407 bp each). Our analysis detected some differences with the sequence reported by us, but changes were located in the *rrnL* variable region and the samples come from different geographical locations (Towler, *et al.*, 2001).

The inferred sizes of *rrnL* and *rrnS* correspond to 1,132 bp and 727 bp, respectively, and are similar to the reported lengths of many other *rrn* genes in Arthropoda.

3.6 Non-coding regions

The total non-coding sequence of the *C. limpidus* mitochondrial genome corresponds to 586 bp. The largest one, the putative control region, encompass a tract of 545 bp located between *rrnS* and *trnQ*. This region contains a lower AT content (60.92%) compared to the rest of the mitochondrial genome (64.46%), an atypical characteristic if compared with others control regions. Within this sequence, several inverted repeats were found. The largest one is a 10 bp inverted repeat [5'-CTCCCCTCCG N_{29} CGGAGGGGAG-3'], but additional 9 bp and 8 bp repeats could be involved in replication or transcription of the genome.

The largest non-coding region excluding the control region has 15 bp and is located between *trnS2* and *nad1*. The rest correspond to spacers up to 5 bp.

3.7 Comparison between the mitochondrial genomes of C. limpidus and M. gibbosus

Recently, the mitochondrial genome sequence of *M. gibbosus* was deposited in GenBank. This scorpion and *C. limpidus* belong to the same family: Buthidae. However, their mitochondrial genomes show some differences: the mtDNA of *M. gibbosus* is 1,162 bp larger than the mtDNA of *C. limpidus*. The additional

DNA is present within the largest non-coding region (D-loop). Remarkably, the *M. gibbosus* D-loop is characterized by the presence of very large repeated elements up to 172 bp in length. The AT content in these two genomes is very similar (Table 2). The identity between the proteins encoded in both genomes range from 49.0% (ATP8) to 71.4% (COB). The identity between their *rrnS* is 65.6%, and for the *rrnL* is 66.4%. The order of protein-encoding genes in the mitochondria of these organisms is the same to that reported in *L. polyphemus*. However, the gene arrangement of the *trn* genes differs slightly between the two scorpions: the *trnN* gene in *C. limpidus* is located between *trnS1* and *trnR*, but in *M. gibbosus* is embedded in the complementary strand of the *rrnS* gene (Figure 2).

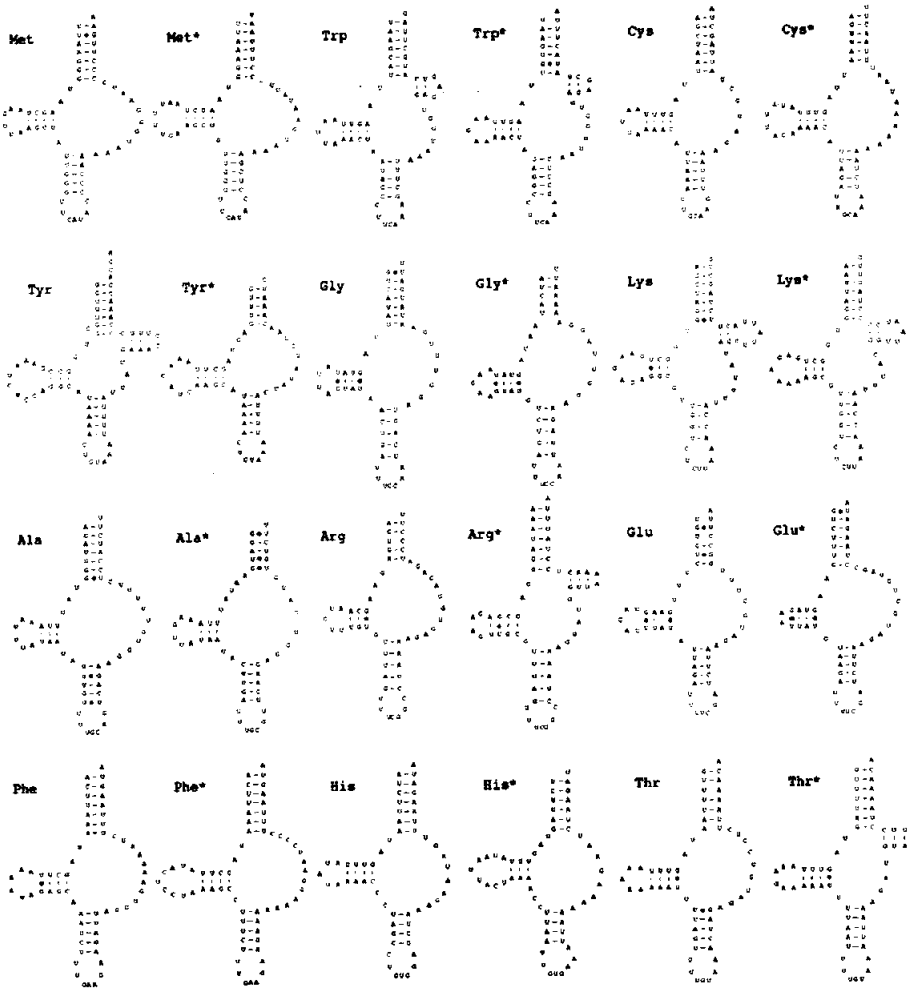


Figure 3. Comparison between mt-tRNAs predicted secondary structures of *C. limpidus* and *M. gibbosus*. Structures belonging to *M. gibbosus* are marked with an asterisk. Bars indicate Watson-Crick base pairings and G-U pairings are pointed out by dots. The enclosed tRNAs structures correspond to the putative products of *trnS1* and *trnI* in *C. limpidus*. The names and typical structures present in the tRNAs are shown in the scheme at the bottom of the figure.

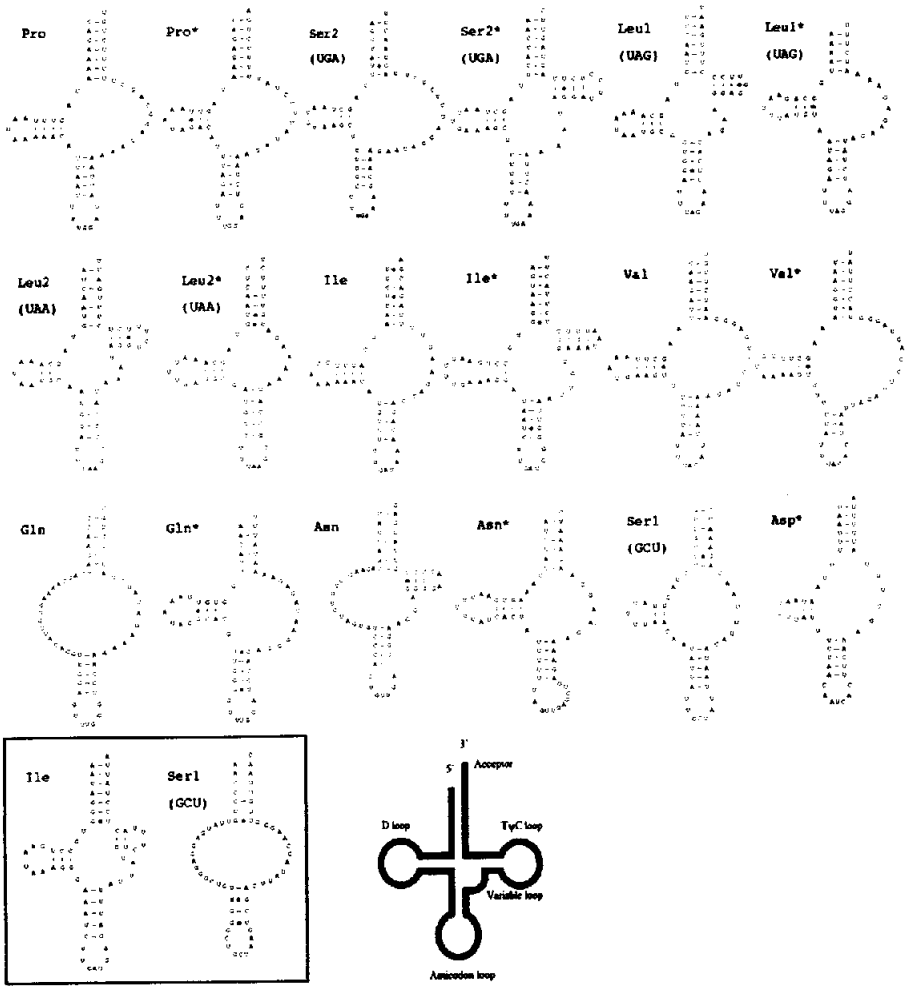


Figure 3 (*continued*)

The same general characteristics observed in the structure of the tRNAs of *C. limpidus* are also present in the *M. gibbosus* tRNAs (Figure 3): they are reduced in size; none of them contain the CCA signature present in the 3'OH of the canonical tRNA. Frequently, their aminoacyl acceptor arms are poorly paired, suggesting that some editing mechanism is required for their functionality (Yokobori and Pääbo, 1995b). Fifteen of their tRNAs contain a TV replacement loop instead of the typical TψC arm. Besides their sequence similarities, some of the *M. gibbosus* tRNAs possess a different structure compared with the equivalent *C. limpidus* tRNA structure, but nevertheless, they have evident sequence similarities, with the anticodon arm being the most conserved. The most conspicuous structural differences between the tRNAs of these scorpions are: the *C. limpidus* $tRNA^{Tyr}$, $tRNA^{Leu2}$ and $tRNA^{Leu1}$ have a TψC loop, but these are absent in the same tRNAs of *M. gibbosus*. Similarly, the *M. gibbosus* $tRNA^{Arg}$, $tRNA^{Thr}$ and $tRNA^{Ser2}$ contain a TψC loop, which is not present in the equivalent tRNAs in *C. limpidus*. The *M. gibbosus* $tRNA^{Gln}$ has a D loop which is missing in the other scorpion. The *C. limpidus* $tRNA^{Asn}$ lacks D and TψC loops, but the D loop is present in this tRNA in

M. gibbosus. These structural changes are the product of a few changes in sequence. As described above, the *trnD* gene has different positions in the genomes of the scorpions analyzed here. Both genes show a very poor similarity, and the structure of their products is also different. Probably this situation only reflects a distinct annotation criterion. Genes encoding $tRNA^{Ile}$ probably suffer from the same problem: they are annotated, in both genomes, in the same relative positions but they are different in sequence and their products also exhibit different structures. Interestingly, an alternative *trnI* gene was located in the complementary strand of *rrnS* gene, a very atypical position. This putative gene and its product share more similarity with its counterpart, in *M. gibbosus*. More experiments are needed to solve these discrepancies.

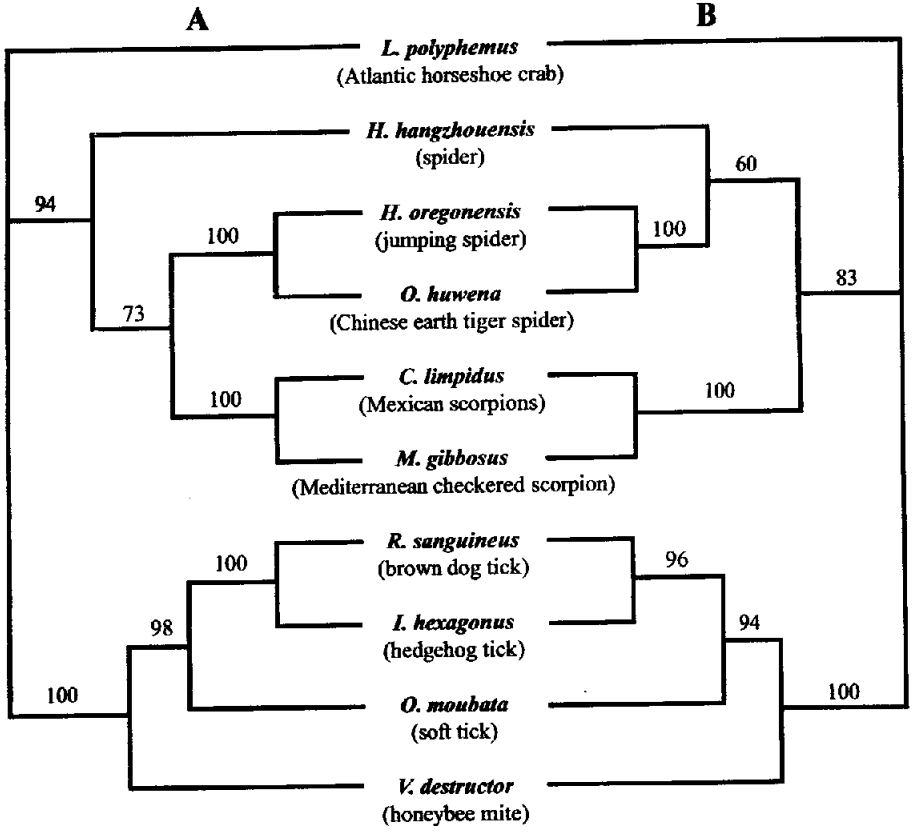


Figure 4 Maximum parsimony unrooted phylogenetic trees obtained with A, ten concatenated proteins (excluding ATP8, NAD2, and NAD6), and B, with four concatenated protein-sequences (COI, COII, COB and NAD1) encoded in the mitochondrial genomes of representative Chelicerata is presented. *L. polyphemus* was used as an out-group. Numbers within the tree are the bootstrap values (1000 replicates). Common names of species are within parenthesis.

3.8 Phylogenetic considerations

To elucidate the phylogenetic position of the *C. limpidus* and *M gibbous* scorpions among the Arachnida, several unrooted trees were obtained using different protein data sets as described in Materials and Methods. This analysis produced a single most parsimonious tree for each data set, all with the same topology. Figure 4A shows the tree generated using the concatenated proteins without ATP8, NAD6 and NAD2. This tree was selected because all its internal branches were supported with high bootstrap values (73-100%). The tree had a

total length of 9,358 steps, a consistency index of 0.7931, and a rescaled index of 0.3479. *C. limpidus* and *M. gibbosus* formed a tight cluster (100% bootstrap value), and also indicates that Scorpiones is a sister group of the spiders (Araneae) *H. oregonensis* and *O. huwena*, with a bootstrap value of 73% and was clearly separated from the Acari (94%). However, the spider *Heptathela hangzhouensis* renders a paraphyletic group (Qiu, *et al*., 2005).

We tested *H. hangzhouensis* for rate homogeneity with the rest of the spiders and found that this lineage has a different rate using the Tajima relative rate test (data not shown). This result was consistent when three different outgroups (*C. limpidus*, *I. hexagonus* and *L. polyphemus*) were used, and it is the probable cause of the apparent paraphyly of the spiders. Nevertheless, a tree constructed using only COI, COII, NAD1 and COB, the most conserved proteins, showed that the scorpion formed a sister group with the spiders (Figure 4B). This tree had a total length of 3,272 steps, a consistency index of 0.7784, and a rescaled index of 0.3573.

The lack of information about the mitochondrial genome sequences of representatives of some important arachnid orders (Uropygi, Amblypygi, Pseudoscorpiones, Solifugae, and Opiliones) prevents the establishment of the correct phylogenetic position of the Scorpiones, however our results do not support this order as a sister group to all the Arachnids, as has been proposed (*i. e.* Weygoldt and Paulus, 1979).

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CAPITULO III

Resumen

Los únicos trabajos sobre las especies del género *Centruroides*, tratan del estudio de su veneno, algunas descripciones morfológicas que incluyen la reevaluación de las especies Sudamericanas, y algunos trabajos moleculares que determinan el parentesco filogenético entre las subespecies de *C. limpidus* y *C. infamatus*, cuya distribución es en la parte central de México. Uno de los trabajos más extensos sobre la clasificación de los *Centruroides* de México fué realizada por Hoffmann y a partir de ahí los distintos grupos dentro de *Centruroides* se han nombrado solo por convención, considerando a los grupos Bertholdii, Thorelli, Gracilis y al grupo de los alacranes Rayados.

En este trabajo se utilizaron datos morfológicos y morfométricos para determinar las relaciones filogenéticas entre las especies mexicanas de *Centruroides*. Hasta la fecha ningún análisis cladista se había realizado en este grupo.

El registro fósil hace suponer que la familia Buthidae, a la que pertenecen los *Centruroides*, tuvo su origen en el continente de Laurasia (actualmente América del Norte, Europa y Asia).

Este género solo se encuentra en el Nuevo Mundo, por lo que se ha considerado que América del Norte y particularmente en México se encuentran las especies de las cuales se diversificaron las demás del género.

Las filogenias obtenidas a partir del método de Máxima Parsimonia, arrojan una filogenia con valores bajos de bootstrap utilizando solo datos morfológicos, con los datos morfométricos los valores aumentan un poco sin embargo, haciendo una combinación de los datos se obtiene una filogenia con valores de bootstrap mejores. Los análisis llamados de evidencia total incluyen todos aquellos datos obtenidos de un grupo, esto permite hacer reconstrucciones filogenéticas más informativas.

Las principales conclusiones obtenidas es que los *Centruroides* están formados por al menos dos clados, mostrados en la filogenia obtenida a partir de la matriz con los datos combinados, uno es Gracilis y el otro Thorelli, ni el grupo Rayado ni el Bertholdii muestran soporte en esta filogenia.

Phylogenetic analysis of scorpions from the genus *Centruroides* Marx, 1890 of Mexico, using morphological and morphometric characters

Sonia Dávila,[1*] Norma A. Valdez-Cruz,[2] Lourival D. Possani[2] and Daniel Piñero[1]

[1]Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Ciudad Universitaria, D. F. México CP 04510

[2]Departamento de Medicina Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad, 2001, Apartado Postal 510-3 Cuernavaca, Morelos, México CP 62210

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Author for correspondence: Sonia Dávila Ramos.

Phone: 52 (777) 329 16 90

Fax: 52 (777) 317 55 81

E-mail: sdavila@miranda.ecologia.unam.mx

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ABSTRACT

The initial work on the classification of the *Centruroides* scorpions of Mexico were based on morphological characters. Recently, it was described a conventional classification of this genus assuming the existence of four groups, namely: Bertholdii, Gracilis, Stripes and Thorellii. Due to the fact that these studies were also conducted taken into consideration only a few morphological features of the scorpions and that some of them are medically important, it is necessary to revise in more depth this classification. Here we used a cladistic approach in order to describe the phylogenetic relationships among Mexican scorpions of the genus *Centruroides*. The analysis was based on 79 morphological and morphometric characters assuming equal weight for each one of them. We used 25 specific terminal taxa plus an additional outgroup, represented by the species *Rhopalurus junceus* from the same Buthidae family. The criterion of "maximal morphological diversity" was chosen for selecting the species. This approach provides a stronger test of monophyly than random selection. The boostrap values obtained from our analysis support the existence of at least two clear clades: Thorellii and Gracilis. The bootstrap values obtained for the group Stripes are acceptable, but that of Bertholdii does not support it as a separated group.

KEY WORDS: Scorpion; *Centruroides*; morphologic characters; morphometric analysis; parsimony; evolution.

INTRODUCTION

The scorpions play an important role for the phylogeny of the chelicerates (Wheeler, 1998). Their wide geographical distribution, local abundance and medical importance make them an adequate model for evolutionary studies (Polis, 1990).

Specific morphological and some molecular features were taken to describe scorpions of various groups, mainly at the level of family or higher taxa (Order or Class) (Prendini 2000, Stockwell, 1988; 1992). Several identification keys have been proposed and used for their classification and systematics. Vachon (1963, 1974) established two main characters: dentition and pedipalp trichobotria for classification of chelicerates at the level of genus and families. Latter, cladist approaches provided better tools for the classification of the scorpions, based on parsimony analysis. This method relies on a mathematical algorithm that allows to compare informative characters, either morphological, morphometric, or more recently including the use of data obtained from molecular biology (nucleotide or amino acids sequences) Lamoral (1980), Francke and Soleglad (1981), Francke (1981, 1982), Laurenco (1985), Stockwell and Levi (1989) and Prendini (2000). These authors have proposed specific phylogenetic hypothesis and taxonomic classifications for the different groups.

Buthidae is the largest family of extant scorpions. In the "Catalog of scorpions of the World" 73 genera and 529 species of the family Buthidae are listed (Fet and Lowe, 2000). Some genera of this family like *Androctonus*, *Buthacus*, *Buthus*, *Centruroides*, *Hottentotta*, *Leiurus*, *Mesobuthus*, *Parabuthus* and *Tityus* include many species with medical importance, distributed all over the world. It is interesting to note that in the New World the diversity is higher than in the Old World (Fet *et al.*, 2000). In America, dangerous species have been found in the Southern part of United States,

Mexico, Eastern South America and in the Caribbean islands (Polis, 1990). All of them belong to the genus *Centruroides*.

Genus *Centruroides* Marx, 1890.

The name *Centruroides* was first introduced by Marx (1890) for two species: *Centruroides exilicauda* (Wood, 1863), and the new species *Centruroides luctifer*. The former became the monotypic species since no description was published about the second one, which was considered as a *nomen nudum*. Marx (1890) used also the name *Centrurus* for the same species, so that the name *Centruroides* was not accepted officially.

The name *Centrurus* Ehrenberg 1829 was incorrectly used for many years to denote the species of *Centruroides* Marx, 1890. However, these two names are not synonymous. The priority in type designation belongs to C. L. Koch (1838), who first used the name in combination with a description of the species, *Centrurus galbineus* C. L. Koch, 1838. Then, *Centrurus* was found to be the junior synonym of *Heterometrus* Ehrenberg, 1828 (Scorpionidae). It is not a *nomen nudum* as cited by Francke (1985) (see also Braunwalder and Fet, 1998).

According to Armas (1988) representatives of the genus *Centruroides*, like the fossil species of *C. beynai*, were found in the Cenozoic fossil record in amber. Schawaller (1979) characterized the species from the Oligocene amber of the Dominican Republic, most probably identical to the extant *C. nitidus*. Another fossil species from Miocene/Oligocene amber of Chiapas, Mexico, probably belongs to the same genus. They were described, but no specific names assigned (Santiago-Blay and Pionar, 1993; Grimaldi, 1996).

Centruroides is a large genus comprising 46 species and 22 subspecies (Fet and Low, 2000). They are found exclusively in the New World with a center of distribution in Central America, Caribbean and Mexico, and extending to Southern regions of United States and the Northern part of South America. The genus includes animals with small sizes but also some large species, living in quite diverse environments: from hot arid deserts to tropical rainforests. In Mexico there are two main biogeographic zones: the neartic and neotropical. This probably produced the generation of a widely variable scorpion fauna. Mexico is considered to be the center of distribution of the American scorpions, with 221 different species of scorpions, 25 of them belonging to the genus *Centruroides*. From these, seven species are toxic to humans (Fet et al, 2000). The species *C. noxius* and *C. limpidus*, possess a potent venom, potentially lethal to humans and constitute a significant public health hazard. A modern comprehensive analysis of the classification of this genus does not exist, reason why this study was undertaken.

The taxonomy of many *Centruroides* species is confused and their systematics has been traditionally based on the features of morph sculptures and coloration characters. There are several generic keys for identification of species of this genus, but no modern taxonomic or phylogenetic analysis has been presented, thus far (Díaz Nájera, 1966; Ibáñez-Bernal, 1995; Pocock, 1894; Stahnke, 1972; Stahnke, and Calos, 1977 and Sissom and Laurenco, 1987).

Hoffmann (1932) made some of the most important classifications of the Mexican *Centruroides* scorpions. Initially he proposed to separate the genus into scorpions without stripes and with stripes. The first group was subdivided in the scorpions with eight granulation series in the cutting edges of the movable finger. This includes *C. flavopictus*, *C. margaritatus*, *C. ochraceus*, *C. bertholdii* and *C. noxius*. The second group includes *C. nigrescens*, *C. nigrimanus*, *C. fulvipes* and *C.gracilis*, have

nine granulation series in the cutting edges of the movable finger. In the second group he also considered the species *C. thorellii* as a separated one, because it has a very small size compared with the others. The remaining species were subdivided in species with 4 longitudinal dark lines in the carapace that includes *C. limpidus, C. elegans* and *C. pallidiceps*, and the species with some dark coloration in the carapace, without lines, that includes *C. infamatus*, *C. suffusus*, *C. vittatus*, *C. exilicauda* and *C. nigrovariatus*.

In 1938 Hoffmann considered additional characteristics, such as geographic distribution and venom toxicity. In this sense he recognized the relations among some species independent from their morphology. He considered that *C. gracilis* and *C. margaritatus* were closely related because of their wide distribution in an extensive zone of Mexico and some countries of South America, due to the easily adaptation to humid climate. In another case, *C. vittatus* and *C. exilicauda* were related because of their geographical distribution in the Northern part of Mexico and due to the low toxicity of their venoms. He classified *C. infamatus*, *C. suffusus*, *C. limpidus*, and *C. elegans*, because they are found in the central part of Mexico. *C. nigrovariatus* was considered a related species, although it was found in the central part of Oaxaca in Mexico. *C. flavopictus* and *C. ochraceus* do not seem to be closely related with the remaining scorpions but he considered that these species are more related to the *C. gracilis* and *C. margaritatus* species. He also showed that the presently known toxic species for humans were rather located in the area that originally was occupied by the meridian peninsula of the ancient North American continent, before bridging with the Southerland areas of the Neotropics.

The most recent classification of the Mexican *Centruroides* genus was based on the conventional classification proposed by Gonzalez (2001). In his study four groups of *Centruroides* scorpions were described: Gracilis, Bertholdii, Thorellii and Stripes.

In this work we present morphologic and morphometric data that support a phylogenetic relationship, which could explain the speciation of the genus *Centruroides* in Mexico. Particularly, we propose a systematic classification of these scorpions. Finally, we describe and characterize twenty-five species of scorpions using cladistic analysis based on two matrices and the integration of the entire set of data.

MATERIALS AND METHODS

Taxa

All twenty-five *Centruroides* species considered from Mexico (Fet et al, 2001) were included in this study. The description of species were obtained either directly from the collected animals in the field or from alcohol-preserved animals from the collection of The American Museum of Natural History in New York and from taxonomic descriptions in the literature (Hoffmann, 1932, 1938; Stahnke, 1972; Stahnke, and Calos, 1977; Sissom 1991a, 1991b, 1995, Armas 1977, 1981, 1988, 1994, 1996; Armas, L. F., de and M. Baez. 1988; Armas, L. F., and H. Contreras. 1981; Armas, L. F; C. R. Beutelspacher, and E. Martín F. 1995; Armas, L. F and L. R. Hernández. 1989). *Rhopalurus junceus* is another genus that belongs to the Buthidae family and was used as outgroup for rooting the trees. The study of each species was performed using at least two independent samples of scorpions. Preferentially we used holotype, alotype and paratype scorpions. The keys published by Hoffmann (1932; 1938), Diaz Najera (1966), Stahnke and Calos, (1977), Sissom (1995), Ibáñez-Bernal (1995), Pocock (1894) and Armas were used to identify the collected scorpions. The eighteen species collected were either frozen at –70 C or preserved in absolute ethanol for subsequent studies. Detailed information about the localities where the animals were collected is available under request. The scorpions are also available for consultation *in situ*.

Characters

We have used characters with the maximum morphological diversity inside each clade, which are also assumed to reflect the maximum genetic diversity. Some of the most commonly used morphological and morphometric characters were choosen such as: the presence of stripes, dentition number in pectinal teeths, size, coloration, among

others (in total 79 total different features). Fifty-one binary characters were taken from morphological variations and 10 unordered multistate characters. Additionally, 18 unordered multistate morphometric characters were selected, of these; three discreet ranks were used and named (small, medium and big). Polymorphic characters were scored as recommended when the polarity of the characters is unknown from previous analyses (Kornet and Turner 1999). Missing data (including unavailable and not applicable data) represent only 5.8% of the entries in the data matrix (Appendix 1).

Cladistic analysis

The program PAUP* 4.0b8 (Swofford, 1998) was used to perform heuristic searches for the most-parsimonious trees. Multistate taxa were interpreted as polymorphisms and all characters were always considered unordered. Morphological and morphometric characters were equally weighted. We assumed that this was justified in the absence of objective criteria for weighting some characters more heavily than others. Equal weighting schemes do, however, carry the assumption of low rates of change in all characters (Felsenstein, 1981). For these analyses, all presented trees were rooted using *Ropalurus junceus* as an outgroup wich was choosen because it belongs to the Buthidae family. Nonparametric bootstrapping with 1000 replicas (Felsenstein, 1985) was performed to evaluate branches in the phylogenetic estimates. The consensus tree retains groups compatible with 50% majority rule consensus. Heuristic searches were run for 100 random addition sequence replicates using TBR branch swapping (Felsenstein, 1985).

The reconstructed trees were obtained with three different matrices: morphological, morphometric and combined data.

RESULTS

The morphologic matrix was performed considering the most variable features present in scorpions, as mentioned in Material and Methods, but excluding the evident plesiomorphic and apomorphic characters. These characters have been used in most of the species description and identification keys (Table 1). The tree obtained with these data was the result of 1000 bootstrap replicas (CI= 0.265, RI= 0.453).

Eighteen quantitative characters were obtained in the morphometric matrix. The size and the relation between height and width measurements from the scorpion bodies were used in previous work for the differentiation of species. We do consider this type of information taxonomically useful (Table 2). Only one tree was obtained with 1000 bootstrap replicas (CI= 0.261, RI= 0.474).

The combined matrix (in total 79 characters) was obtained using all morphological and morphometric characters. When we searched the most parsimonious tree, with 1000 bootstrap replicas, thirteen trees with equal values were obtained. The consensus tree (CI=0.287, RI=0.539) for the combined matrices is shown in Figure 1C, whereas the two previous one in Figure 1A and Figure 1B.

The first tree (Figure 1A) presents the Thorellii's group named by Gonzalez (2001) in three separated branches. In the first branch we found the *C. rileyi*, *C. schmidti*, and *C. thorellii* species, in another branch we found the *C. hoffmanni* and *C. sissomi* species and in the last branch appeared *C. chamulaensis*. This group includes the scorpions with smaller body size. Other characters shared by this group are: the general color (yellow with many dark spots in dorsal carapace, mesosoma, pedipalps and legs) and subaculear tubercle that is conspicuous and arise in a ventral keel like a crest.

The second group named by Gonzales as Gracilis, is formed by *C. fulvipes*, *C. gracilis*, *C. nigrimanus*, and *C. nigrescens*. It is well conserved and forms a well-defined branch in this comparison.

The last group defined in Figure 1A is the Stripes group, which is formed by *C. exilicauda*, *C. sculpturatus*, *C. vittatus*, in one branch; *C. infamatus* and *C. nigrovatriatus* in another and *C. suffusus*, *C. elegans* and *C. pallidiceps* in a third branch. The *C. limpidus* species appeared in a homoplasyc branch. The scorpions are characterized by their color patterns and two longitudinal stripes in the dorsal mesosoma. As we can see the Bertholdii named group that includes *C. bertholdii*, *C. noxius*, *C. flavopictus*, *C. ochraceus*, *C. chiapanensis* and *C. margaritatus* species was integrated in the other groups defined.

Figure 1B shows the comparison of the morphometric data, in which better defined groups of species seems to be present compared to Figure 1A. The morphometric analysis shows three principal clades. In the first branch we found all the species belonging to the Thorellii's group and two more species from the Bertholdii group (*C. bertholdii* and *C. noxius*). The second branch includes all the species from the Gracilis group and again two more species from the Bertholdii group (*C. margaritatus* and *C. chiapanensis*). In the last branch we observe six of the eight species that form the Stripes previously defined group and *C. flavopictus* from the Bertholdii's group. The remaining species from this Stripes group map in separated branches.

Combinatory analysis of morphological and morphometric data is presented in Figure 1C. The phylogenetic tree shows three groups. The first is defined by the Thorellii group, formed by *C. bertholdii*, *C. chamulaensis*, *C. rileyi*, *C. schmidti*, *C. thorellii*, *C. hoffmanni*, *C. sissomi* and *C. noxius*. The second group (Gracilis) is determined by *C. chiapanensis*, *C. nigrimanus*, *C. fulvipes*, *C. gracilis*, *C. nigrescens*

and *C. margaritatus*. The last group is formed by *C. exilicauda*, *C. sculpturatus*, *C. flavopictus*, *C. limpidus*, *C. nigrovariatus*, *C. elegans*, *C. infamatus* and *C. suffusus*. The species *C. pallidiceps*, *C. vittatus* and *C. ochraceus*, are located in separated branches.

The overall distribution of genus *Centruroides* of Mexico is shown Figure 2. Twenty-five species were collected in the regions indicated on the map. Each one of the species is represented by numbers and their corresponding groups are shown by different colors.

DISCUSSION

The results here described, clearly show the presence of three distinct groups for the *Centruroides* species of scorpions found in Mexico: Torelli, Gracilis and Stripes as earlier proposed by Gonzales (2001). Unfortunately the fourth group (Bertholdii) defined by Gonzales (2001) was not confirmed in the present analysis.

The clade encompassing the Gracilis and the Stripes groups of species in the three phylogenetic trees of Figure 1 is quite consistent. They are all located in close proximity in the entire analysis. The Torelli group is more disperse in the cladogram of Figure 1A, but again it is quite consistent in the two other phylogenetic trees (Figure 1B and Figure 1C).

Within the Thorellii's group we find *C. chamulaensis*, *C. rileyi*, *C. schmidti*, *C. thorellii*, *C. hoffmanni* and *C. sissomi*. This classification is the best supported by boostrap values in the three cladograms of Figure 1. Interestingly, all these species are arboreous, living in wooden trees. These species are mainly located in the Southern region of Mexico. *C. chamulaensis* and *C. hoffmanni* are found in Chiapas; *C. sissomi* and *C. schmidti* in Quintana Roo and *C. thorellii* in Yucatan. The only species that has

been located in the North part of the country is *C. rileyi* from San Luis Potosi and Tamaulipas (Figure 2). *C. bertholdii* and *C. noxius* are also included in the same group, despite the fact that earlier classifications have included them in the Bertholdii's group (Hoffmann 1932, 1938; Gonzalez 2001). When the results of the morphological tree (Figure 1A) are compared with the morphometric ones (Figure 1B) these two species consistently form a clade with *C. hoffmanni* and *C. sissomi*. This is probably due to the size of the scorpions, which are quite similar to the other species of the same group Thorellii. The species *C. thorellii* of the present classification for some time was the cause of controversy. In 1932 Hoffmann said that this species was found in Mexico, but Sissom (1995) claimed that it was from Guatemala. Almost at the same time, Armas et al (1995) defined four new related species: *C. schmidti*, *C. sissomi*, *C. hoffmanni* and *C. rileyi*. All these were considered to be *C. thorellii* species, due to their great similarities. As shown in the present analysis, they are all *bona fide* independent species and are distributed in Southern part of Mexico. Two other species: *C. sissomi* and *C. chamulaensis* were difficult to classify, due to their resemblance. Finally, thanks to a closer comparative observation, it was possible to establish a clear differentiation between them. *C. chamulaensis* has smaller total size, the subaculear tooth is more developed, the manus is smaller and globular, the body has more frequently distributed granulation than the species *C. sissomi*. Based on the morphological tree (Figure 1A), *C. chamulaensis* is closely positioned to *C. flavopictus*. This is probably the reason why they were earlier considered to be a race of *C. flavopictus*. However, they are smaller size, the number of pectinal tooth and some other distinct characters provided the arguments for Armas (1995) to re-classify the subspecies *C. flavopictus chamulaensis* as simply *C. chamulaensis* species. Thus, the present analysis helps clarifying the disputes on these species and locations.

The Gracilis group comprises the species *C. nigrimanus*, *C. fulvipes*, *C. gracilis*, *C. nigrescens*, *C. chiapanensis* and *C. margaritatus*, because these species appear in the same clade of the three trees with high bootstrap values (Figures 1A-1C). *C. chiapanensis* and *C. margaritatus* form a consistent clade within this group, but earlier were classified in the now eliminated Bertholdii's group. The Gracilis group contains nine granulations series in the internal face of movable finger of the pedipalp, although these characteristics are also present in scorpions of the Torellli's group (*C. rileyi*, *C. schmidti*) or Stripes group (*C. suffusus*). Hoffmann in 1938 considered *C. nigrescens* and *C. fulvipes* as races from *C. nigrimanus* species, because they show slightly different color patterns. However, based in the present analysis they all should be considered as terminal taxa. It is worth noting that in the morphologic tree, *C. ochraceus* appears to share resemblances with the Gracilis group, but in the two others trees the separation is obvious. Maybe this explains why sometimes *C. ochraceus* has been confused with *C. margaritatus*. These species are morphologically similar but several characters like the size and some color patters clearly differentiate them. Also, *C. chiapanensis* was earlier considered as sub-species of *C. margaritatus*, but now are clearly considered independent species. As shown in Figure 2, the distribution of the scorpions of the group Gracilis is widespread in the states of Guerrero Oaxaca, Chiapas, Central part of the country and parts of the Gulf of Mexico. *C. gracilis* and *C. margaritatus* are the widest distributed species, including some other countries of Central and South America. The combined tree shows this clade as a well separated group and probably the most ancient group in the genus.

Within the Stripes group we find: *C. exilicauda*, *C. flavopictus*, *C. limpidus*, *C. nigrovariatus*, *C. elegans*, *C. infamatus*, *C. suffusus*, *C. pallidiceps* and *C. vittatus*. Nine out of the twenty-five species of *Centruroides* in Mexico have been included in the

Stripes group. The intra and interspecific variations for the characters that distinguish these species is very high and overlaps very frequently among the species. The Stripes group is considered an active evolving group formed by closely related morphological species. It is difficult to differentiate species from races (Hoffmann 1938). In this group there is a clade formed by *C. elegans*, *C. infamatus* and *C. suffusus*. *C. suffusus* is found in Durango whereas *C. elegans* is found in several States of the Pacific Coast and Central part of Mexico (see Figure 2). The third species *C. infamatus* is found in many states of the country (see Figure 2). *C. elegans* and *C. infamatus* have a wide distribution range, probably meaning that they are ancestral species. The next clade involves *C. flavopictus*, *C. limpidus* and *C. nigrovariatus*, from which the two last ones are included in the Stripes group, whereas *C. flavopictus* belongs to the now eliminated Bertholdii's group. The geographic distribution of *C. flavopictus* includes the States of Veracruz and Chiapas while *C. nigrovariatus* has been described in Oaxaca. *C. limpidus* has been collected in Colima, Guerrero, Jalisco, State of Mexico, Michoacan, Morelos, Oaxaca and Puebla and shares many geographical areas with *C. infamatus* and *C. elegans*. *C. exilicauda* is morphologically related with *C. sculpturatus*, although in the morphometric tree they are separated species. A recent publication shows that they are indeed two different species (Valdez-Cruz et al, 2004). Two other species considered within this group are *C. pallidiceps* and *C. vittatus*. *C. vittatus* often is misled with *C. exilicauda* and *C. sculpturatus*.

From the twenty-five species of *Centruroides*, two of them: *C. sculpturatus and C. ochraceus* were not included in any of the three groups described here. The first was equivocally described as *C. exilicauda*, the second one *(C. ochraceus)* was misled with *C. margaritatus*.

Finally, the fourth group described by Gonzalez as Bertholdii did not form a clade in our analysis (Figure 1). *C. bertholldi* and *C. noxius* clearly form a clade with the Thorellii group, *C. flavopictus* appears more related with the Stripes group, at least in the morphometric and the combined data analysis, *C. chiapanensis* and *C. margaritatus* are considered inside the Gracilis group. *C. ochraceus* appears, in the morphological tree, to be related with the Gracilis group but, in the morphometric and combined trees it is very distantly related to all the species from this group.

Figure 2 shows the location of all *Centruroides* species reported and their distribution in Mexico. The Stripes group is distributed principally in the North and Central part of the country, and represents the most diverse and abundant group of *Centruroides*. The Gracilis group is also abundant and their distribution is mainly confined to the South part of Mexico including both coastal areas. The Thorellii group is represented by few and not so abundant species. Their distribution is basically in the Southeast part of the country, with only two species situated more to the North. As we can see, the three groups of *Centruroides* are distributed into well-defined areas of the country, possibly indicating specific speciation events, but often they share the occupancy of territory, probably because they have a common evolutionary history.

In conclusion, this work describes the presence of only three groups of scorpions *Centruroides* in Mexico: Thorellii, characterized by its small size and limited abundance; Gracilis, the most abundant and extended species; Stripes that include most of them suggesting the presence of an on-going active recent speciation phenomenum. The Bertoldi group was eliminated by our analysis.

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Table 1 Distribution of 61 morphologic characters among the exemplar taxa chosen for the phylogenetic analysis of the genus *Centruroides* (Marx 1890).

Number of character	1234567890123456789012345678901234567890123456789012345678901
C. bertholldi	1110000000100000000001000011 0?0111100010121111111111010202120
C. chamulaensis	0000110000110010101011101110?0100000111200111111000110202121
C. chiapanensis	111001011010000010001100001 0?11111000015?1100002000021202001
C. elegans	0001010110100222110000000111000100011101211000001000100102030
C. exilicauda	000000010010022222021100011110000001110221111111000011100??0
C. flavopictus	1000010010100110101111101110?000000010021111001120001102 02121
C. fulvipes	1110000110101011100011110110?11111000000 5?1011111000011002121
C. gracilis	2100000000101111100110100110?0110000010042001111100000010 2001
C. hoffmanni	011010?????0?????1??1111??0?111??000?1?2??0?????11102210212?
C. infamatus	0110110011100000000010001111101111111101210011111001110111020
C. limpidus	3101010111100110000010001111000000111102111111111200010010 2130
C. margaritatus	211000011111000110001110 0??0?0101100010142001111100001020 2130
C. nigrescens	1110010101101111111001000001 0?1111110010042?1111110000111 0?12?
C. nigrimanus	1110000111111001110011110??0?01111000001??110000211101020 2111
C. nigrovariatus	1110110000000111111110001??10010001111011011111120001102 12120
C. noxius	3110000000100000000001111011 0?000000100021011111111000010102111
C. ochraceus	31100001101000001100111001107010010000013100011120000101 02000
C. pallidiceps	0111010111100001100011100111001101111201101101111000110202030
C. rileyi	010011000000100000101?001110?111111111120001111110011102 12110
C. schmidti	010011000000100000111?001110?0110011111200011111100111021 2020
C. sculpturatus	0000010000000222220211100110?01000011102210111111000110102021
C. sissomi	01?01???????0000001??1111???011????111?00?0????111?21???? 1
C. suffusus	0110011100001000010000000011100100111010211001111000011101110
C. thorellii	001011000010000000111000111 0?0110011111200011111100111021 2020
C. vittatus	111000000000022211011110011 1?01000000101310111111000000001010
R. junceus	0110000111100000100010001110?001000001101000111120000002 00??0

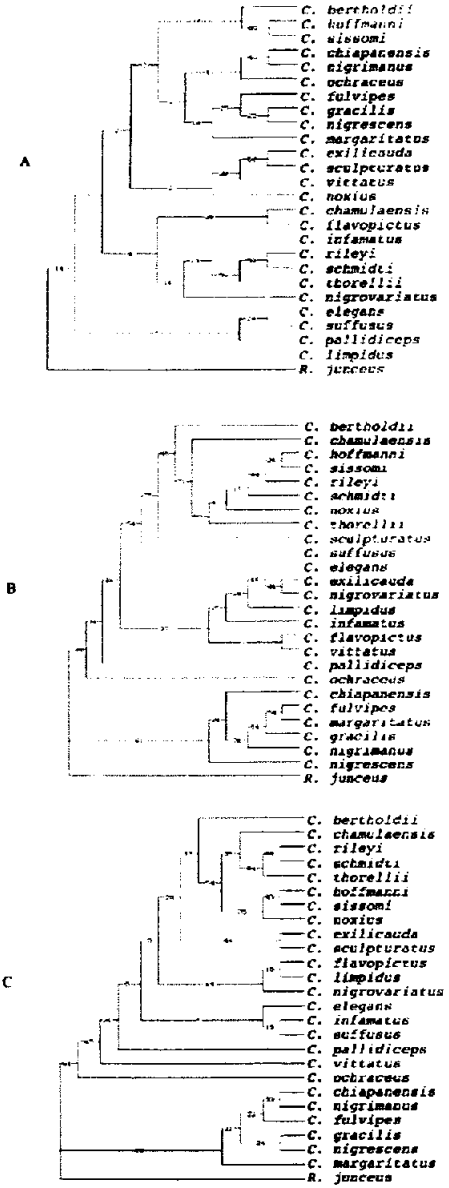
Table 2 Distribution of 18 morphometric characters among the exemplar taxa chosen for the phylogenetic analysis of the genus *Centruroides* (Marx 1890).

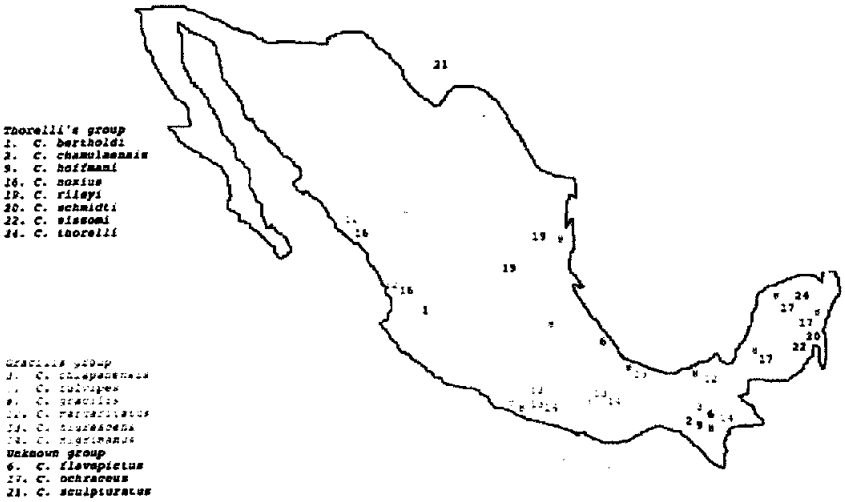
Number of character	123456789012345678
C. bertholdiii	100102110110000010
C. chamulaensis	000101000100000010
C. chiapanensis	222122222222112222
C. elegans	111021100101111111
C. exilicauda	010022100100110111
C. flavopictus	111021111111111111
C. fulvipes	222200222222012221
C. gracilis	222200222222012222
C. hoffmanni	000022000000000000
C. infamatus	101021101111111111
C. limpidus	111022101101111111
C. margaritatus	222200222222022221
C. nigrescens	222122222222222222
C. nigrimanus	222201222222122222
C. nigrovariatus	110022100100111111
C. noxius	000022000100000000
C. ochraceus	111122111112121111
C. pallidiceps	111122111111111112
C. rileyi	000012000000000000
C. schmidthi	000012000100000000
C. sculpturatus	001100110111000110
C. sissomi	000022200000000000
C. suffusus	111101111110001111
C. thorellii	000001000100000000
C. vittatus	111021111111111111
R. junceus	222121111112222222

Legends and Figures

Fig 1 Consensus tree obtained by 1000 replicas of bootstrap. Morphological trees (A), Morphometric trees (B) and Combined data trees (C).

Fig 2 Actual geographic distribution of the *Centruroides* species from Mexico, the number is related with the species and the color is related with the group that the species belong.


A
C. bertholdii
C. hoffmanni
C. sissomi
C. chiapanensis
C. nigrimanus
C. ochraceus
C. fulvipes
C. gracilis
C. nigrescens
C. margaritatus
C. exilicauda
C. sculpturatus
C. vittatus
C. noxius
C. chamulaensis
C. flavopictus
C. infamatus
C. rileyi
C. schmidti
C. thorellii
C. nigrovariatus
C. elegans
C. suffusus
C. pallidiceps
C. limpidus
R. junceus
B
C. bertholdii
C. chamulaensis
C. hoffmanni
C. sissomi
C. rileyi
C. schmidti
C. noxius
C. thorellii
C. sculpturatus
C. suffusus
C. elegans
C. exilicauda
C. nigrovariatus
C. limpidus
C. infamatus
C. flavopictus
C. vittatus
C. pallidiceps
C. ochraceus
C. chiapanensis
C. fulvipes
C. margaritatus
C. gracilis
C. nigrimanus
C. nigrescens
R. junceus
C
C. bertholdii
C. chamulaensis
C. rileyi
C. schmidti
C. thorellii
C. hoffmanni
C. sissomi
C. noxius
C. exilicauda
C. sculpturatus
C. flavopictus
C. limpidus
C. nigrovariatus
C. elegans
C. infamatus
C. suffusus
C. pallidiceps
C. vittatus
C. ochraceus
C. chiapanensis
C. nigrimanus
C. fulvipes
C. gracilis
C. nigrescens
C. margaritatus
R. junceus


Thorelli's group
1. C. bertholdi
2. C. chamulaensis
3. C. hoffmani
16. C. saxius
18. C. rileyi
20. C. schmidti
22. C. wissomi
24. C. thorelli
Unknown group
6. C. flavapictus
17. C. ochraceus
21. C. sculpturatus

APPENDIX 1 Morphologic characters description.

General characters

1 General color: yellow(0); blackish(1); reddish(2).

Carapace

2 Granulations: absent(0); present(1).
3 Interkeels granulations: absent(0); present(1).
4 With 4 longitudinal dark lines: absent(0); present(1).
5 Dark spots: absent(0); present(1).
6 Antero-lateral board edging: absent(0); present(1).
7 Black interocular triangle: absent(0); present(1).
8 Keels: absent(0); present(1).
9 Anterior median keels: absent(0); present(1).
10 Central lateral keels: absent(0); present(1).
11 Posterior median keels: absent(0); present(1).
12 Posterior lateral keels: absent(0); present(1).

Pedipalp

13 Number of primary finger rows: 8(0); 9(1).
14 Femur coloration: lighter(0); darker(1).
15 Patella coloration: lighter(0); darker(1).
16 Tibia coloration: lighter(0); darker(1).
17 Tarsus coloration: lighter(0); darker(1).
18 Top tarsus coloration: lighter(0); darker(1).
19 Dark spots: absent(0); present(1).

Legs

20 Coloration: lighter(0); darker(1).
21 Tibia hairy: absent(0); present(1).
22 Femur keels: absent(0); present(1).
23 Patella keels: absent(0); present(1).
24 Tibia and tarsus keels: absent(0); present(1).
25 Dark spots: absent(0); present(1).
26 Tibia spur: absent(0); present(1).
27 Pedal spur: absent(0); present(1).

Dorsal mesosoma

28 Two black longitudinal bands: absent(0); present(1).
29 Bands: discontinuous(0); continuous(1).
30 Median keels tergite 1: absent(0); present(1).
31 Median keels tergite 2-6: absent(0); present(1).
32 4 keels in tergite 7: absent(0); present(1).
33 Lateral keels tergite 1-2: not granulate(0); granulate(1).
34 Lateral keels tergite 3-6: absent(0); present(1).
35 Lateral board edging in the dorsal tergites: absent(0); present(1).
36 Lateral longitudinal yellow bands: absent(0); present(1).
37 Medial longitudinal yellow band: absent(0); present(1).
38 Color of tergite 7: lighter(0); same color(1); darker(2).
39 Dark spots in dorsal tergites: absent(0); present(1).

Ventral mesosoma

40 Color of the pecten teeth: lighter(0); darker(1).
41 Number of pecten teeth in female: 12-17(0); 18-20(1); 21-23(2); 24-26(3); 27-29(4); 30-39(5).
42 Number of pecten teeth in male: 15-22(0); 23-27(1); 28-32(2); 33-37(3); 38-40(4).

43 Central hole in the basal piece: absent(0); present(1).
44 Genital operculum color: lighter(0); darker(1).
45 Color of ventral tergite 1: lighter(0); darker(1).
46 Color of ventral tergite 2: lighter(0); darker(1).
47 Color of ventral tergite 3: lighter(0); darker(1).
48 Color of ventral tergite 4: lighter(0); darker(1).
49 Color of ventral tergite 5: lighter(0); same color(1); darker(2).
50 Ventral tergite 1: not granulate(0); granulate(1).
51 Ventral tergite 2-4: not granulate(0); granulate(1).
52 Ventral tergite 5: not granulate(0); granulate(1).
53 Dark spots in ventral tergites: absent(0); present(1).

Metasoma

-Postabdomen

54 Keels segments 1-4: not granulate(0); light granulate(1); strong granulate(2).
55 Keels segment 5: not granulate(0); light granulate(1); strong granulate(2).
56 Color segment 5: lighter(0); same color(1); darker(2).
57 Dark spots: absent(0); present(1).

-Telson

58 Subaculear tubercle development: without(0); little development(1); very development(2).
59 Subaculear tubercle origin: away from base of sting(0); close from base of sting(1).
60 Inclination of the subaculear tooth: not to the sting(0); to the first half of the sting(1); to the half of the sting(2); to the second half of the sting(3).
61 Vesicle granulations: absent(0); present(1).

Morphometric character description

1 Carapace-Length: small(0); medium(1); large(2).
2 Mesosoma-Length: small(0); medium(1); large(2).
3 Metasoma-Length: small(0); medium(1); large(2).
4 Total-Length: small(0); medium(1); large(2).
5 Rel. Met/Total: small(0); medium(1); large(2).
6 Rel. Car/Met: small(0); medium(1); large(2).
7 Segment I-Length: small(0); medium(1); large(2).
8 Segment II-Length: small(0); medium(1); large(2).
9 Segment III-Length: small(0); medium(1); large(2).
10 Segment IV-Length: small(0); medium(1); large(2).
11 Segment V-Length: small(0); medium(1); large(2).
12 Vesicle-Length: small(0); medium(1); large(2).
13 Acuelus-Length: small(0); medium(1); large(2).
14 Telson-Length: small(0); medium(1); large(2).
15 Pedipalp Femur-Length: small(0); medium(1); large(2).
16 Pedipalp Patella-Length: small(0); medium(1); large(2).
17 Pedipalp Manus-Length: small(0); medium(1); large(2).
18 Pedipalp Movable Finger-Length: small(0); medium(1); large(2).

CAPITULO IV

Resumen

Pocos trabajos han utilizado herramientas moleculares para determinar relaciones filogenéticas dentro de los alacranes. En este artículo se presenta la filogenia obtenida a partir de los genes mitocondriales COI y COII en el género *Centruroides*. Los organismos utilizados como grupos externos fueron: el alacrán, *Mesobuthus gibbosus*, que pertenece a la misma familia Buthidae; la araña, *Habronattus oregonensis* y el xiphosuro *Limulus polyphemus*. Los resultados principales que se muestran en el árbol obtenido con la combinación de los genes, son:

El género *Centruroides* forma un grupo monofilético.

La topología obtenida presenta dos clados principales ((*C. baergi* + *C. margaritatus*) + (*C. fulvipes* + (*C. pallidiceps* + *C. ochraceus*))) y ((*C. suffusus* + (*C. thorelli* + *C. elegans*)) + (*C. flavopictus* + *C. exilicauda*)), sustentados por distintos métodos de reconstrucción filogenética, así como por valores de bootstrap y quartet puzzling consistentes.

Las especies restantes del análisis forman grupos hermanos, algunos bien sustentados, de los dos clados principales.

Por último, en el árbol se observa que ninguna de las especies "peligrosas" ó tóxicas para los humanos, estan presentes en el primer clado, sin embargo estas especies se encuentran distribuidas tanto en el segundo clado como en las ramas de los grupos hermanos. Esta dispersión de las especies peligrosas a lo largo del árbol sugiere que la toxicidad de los venenos se originó más de una vez en la historia evolutiva de los alacranes.

Phylogenetic Analysis of Genus *Centruroides* Based on Mitochondrial Cytochrome Oxidase I and II Genes

Sonia Dávila[1], Alejandra Vázquez-Lobo[1], Guillermo Dávila[2], Alexei Licea[3] and Daniel Piñero[1]*.

1 Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Apartado Postal 70-275, Ciudad Universitaria, C. P. 04510, D. F., México.
2 Programa de Genómica Evolutiva, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México. Apartado Postal 565-A, Cuernavaca, Morelos, México.
3 Laboratorio de Inmunología Molecular y Biotoxinas, Departamento de Biotecnología Marina, C.I.C.E.S.E. Km. 107 Carretera Tijuana - Ensenada C. P. 22860, Ensenada, B.C. México.

*Corresponding author:
Daniel Piñero, Ph.D.
Instituto de Ecología – UNAM
Ciudad Universitaria, México D.F.
Apartado Postal 70-275
Codigo Postal 04510
México
Tel. +52-55-56229001
FAX: +52-55-56228995
E-mail: pinero@miranda.ecologia.unam.mx

Abstract

The genus *Centruroides* is one of the 73 genera from the most diverse family of scorpions named Buthidae. The genus includes 46 species distributed in America. We explored for the first time the molecular phylogenetic relationships of 19 of the species, mostly from Mexico (including the toxic species for humans in this genus).
The mitochondria protein-coding genes cytochrome c oxidase subunit I (COI) and subunit II (COII) were chosen in order to assess the molecular systematic relations within the group. In a combined analysis 2208 characters were obtained for 19 ingroup taxa and 3 outgrups, these were analyzed with maximum parsimony, maximum likelihood and bayesian methods. All analyses result in a nearly fully resolved topology within species groups, the monophyly of the genus Centruroides was well supported.

Keywords: Cytochrome *c* oxidase, *Centruroides*, Parsimony, Maximum Likelihood, MrBayes, Phylogeny

Abbreviations: C, *Centruroides*; **COI-COII**, cytochrome *c* oxidase subunits I-II

Introduction

Scorpiones is a very ancient order of Arthropods, often referred to as "living fossils" the first to emerge from an aquatic environment onto land during the Silurian period about 425-450 MYA (million years ago) (Kjellesvig-Waering, 1986). Study of fossil specimens has shown that they have survived with a little change (Ennik, 1972). According to Armas and Baez (1988) representatives of the genus *Centruroides*, like the fossil species of *C. beynai*, were found in the Cenozoic (among 20 MYA) fossil record in amber. Schawaller (1979) characterized the specimens presented in Oligocene amber from the Dominican Republic, most probably very similar to the existent *C. nitidus*. Another fossil species from Miocene/Oligocene amber from Chiapas, Mexico, probably belongs to the same genus. They were described, but no specific names assigned (Santiago-Blay and Pionar, 1993; Grimaldi, 1996).

Scorpions are among the best known invertebrate organisms. Several synthetic works on their basic ecology, evolution, genetics, physiology and immunology have been published (Pocock, 1893; Possani, 1984; Hadley, 1990; Polis, 1990; Nenilin and Fet, 1992).

The genus *Centruroides* Marx 1890 is the most diverse within the largest family of scorpions (Buthidae) and is widely distributed in the New World: North America, the Antilles, Central and South America (Stahnke and Calos, 1977; Sissom and Lourenco, 1987). In North America, all species dangerous to humans belong to the genus *Centruroides* (Polis, 1990).

Worldwide taxonomic, life history, and the medical importance mainly in the genus *Centruroides*, have been relatively well documented (Stanhnke 1978). This strong

scientific foundation, has led to the use of *Centruroides* species in hundreds of basic studies in a wide variety of biological disciplines (Bond, 1992; Armas *et al.*, 1995).

Despite the extensive work on *Centruroides* species, the phylogenetic framework within which to interpret these studies remains weak. The current classifications and hypotheses of phylogeny among the different species of *Centruroides* genus are examined here and a framework for a revision of their classification is presented. Because of their importance to the establishment of the monophyly of the *Centruroides*, the problems of the classification and hypotheses of phylogenetic relationships among the species are also addressed.

The mitochondrial cytochrome oxidase I and II (coding genes COI and COII) may be useful for exploring relationships at or below the generic level (Caterino and Sperling, 1999; Frati *et al.*, 1997). These two proteins coding genes, particularly COI, show excellent performance in resolving relationships at the level of species among *Centruroides*, 19 *Centruroides* species, were used to infer their phylogenetic relationships.

Materials and methods

Sampling

The specimens used in this study were obtained in the field, from live laboratory cultures, and from alcohol-preserved private and museum collections (Table 1). They represent 19 of the 46 species of *Centruroides* described by Fet, *et al* (2000). It was almost impossible to collect the rest of the species, because they are not high abundance and well distributed, and the specimens finding in the museums are not in their better conditions. Most of them belong to the non-continental species distributed in the Caribbean islands.

Molecular techniques

Total genomic DNA was extracted from the complete organism homogenized with liquid nitrogen using the Dneasy Plant MiniKit (Qiagen). The manufacturer protocol was followed with small modifications, to improve yield of mitochondrial DNA.

To obtain the sequence of COI and COII, two overlapping amplification reactions were made. The first fragment included the COI-NH2 (amino) terminal and the second the COI--COOH (carboxyl) terminal and COII complete sequence, the size of the two fragments was around 1 kb. Primers were designed using the mitochondrial sequence from *C. limpidus* (AY803353); C15F04 (22mer) 5'-AGTTTTATGCCTGGAGATTTCG-3', C13R04 (22mer) 5'-TACCAGCCAAAATAGCAAACAC-3', C25F04 (22mer) 5'-AGGGTTGACTGGTGTTATTTTG-3', C23R04 (22mer) 5'-CATTTGAGGCACCAGCTAGAAG-3'. Amplification reactions included 0.2mM of each dNTP, 0.2mM of each primer, 1X PCR Buffer, 1µl of total DNA, and 1 unit of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) in a 50µl volume. DNA

amplification was performed by 1 cycle of 5 min at 94 °C and 35 cycles of 30 s at 94 °C, 45 s at 60 °C and 90 s at 68 °C with a final extension step of 72 °C for 10 min. In all cases, the polymerase was added at the end of the initial denaturation step. The amplification products were separated by electrophoresis on 1% agarose gels to estimate their size and concentration. The fragments were purified from gel using the QIAquick ™ Gel Extraction Kit (Qiagen), then, they were cloned into the pZero vector (Invitrogen) and transformed into *E. coli* (DH5α). The selected clones include one of the two PCR fragments from each species. Each insert was sequenced from both ends using the universal primers of the pZERO vector. The sequencing reactions were made using the BigDye Terminator Cycle Sequencing Ready Reaction kit and run in an AbiPrism 3700 DNA Sequencing System (Applied Biosystems).

Phylogenetic analyses

Our collection consisted of 19 sequences from *Centruroides* spp and three from outgroups (the scorpion *Mesobuthus gibbosus*, the spider *Habronattus oregonensis* and the horseshoe crab *Limulus polyphemus*).

Experimentally obtained sequences were assembled using the computer software phred_phrap (http://bozeman.mbt.washington.edu/phredphrapconsed.html; Ewing, *et al.*, 1998) and deposited in GenBank; Table 1.

Orthologous sequences were then aligned, using the CLUSTAL W default settings (Thompson, 2003) and further refined manually using the Se-Al v2.0a11 program (Rambaut, 2002). Three different alignments were obtained, one of each gene COI and COII and the combined that contains the concatenated two genes. The alignments were analyzed with MEGA v. 2.1 (Kumar, *et al.*, 1993).

Given that the genes used are known to present high variability, we performed a preliminary analysis of the degree of gene saturation by examining the relationships of uncorrected p distances with the number of substitutions (transitions and transversions separately) between species pairs. We did this separately for first, second and third codon positions. Codon position assignment and distance data were obtained using MEGA package.

Phylogenetic trees of the sequences were constructed by maximum parsimony (MP) and maximum likelihood (ML) methods using PAUP* version 4.0b10 (Swofford, 1998). All phylogenetically uninformative sites were ignored and gaps were considered as missing data. MP analyses were performed using heuristic searches with 10 random stepwise additions of taxa (TBR branch swapping, MulTrees option in effect). For ML analyses, the best-fit model of nucleotide substitution for our data was selected by statistical comparisons of 56 different models of evolution with the program Modeltest version 3.06 (Posada and Crandall, 1998) using the Akaike information criterion (AIC). Robustness of the inferred MP tree was tested by bootstrapping (Felsenstein, 1985) with 100 bootstrap replicates each with 10 replicas of random addition. Robustness of the ML analyses was tested by quartet puzzling (QP) with 1000 puzzling steps.

A Bayesian inference (BI) of *Centruroides* phylogeny was performed with MrBayes 3.0 (Huelsenbeck and Ronquist, 2001) by Metropolis coupled Markov chain Monte Carlo (MCMCMC) sampling for 2,000,000 generations (four simultaneous MC chains; sample frequency 100; burn-in 100,000 generations and 0.1 of heating temperature) under the GTR model.

Results and discussion

Sequence Variation and Saturation

In the individual and combined alignments, it shown that all overlapping regions were contiguous and no frameshifts or nonsense codons were apparent. We reasonably assumed that these sequences were only of mitochondrial origin. Nucleotide variation in mitochondrial COI (1530bp) and COII (678bp) genes showed an adenine-thymine *bias* in their nucleotide composition (A+T = 62% for COI; A+T = 63.4% for COII) that is consistent with other arthropod mitochondrial genomes (Boore, 1999), with an overall transition/transversion ratio of 1.39.

The two genes used in this study (COI and COII), are different in their structure, but they share some traits in common like the same location in the genome or the function of the proteins that they encode. The ILD test results suggest that the COI-COII partitions were incongruent. A significance level of P = 0.001 suggest that the data reject the null hypotheses that the partitions in question 1 have the same history and 2 cause insignificant levels of systematic error. Even that if we put only the species that are consistently supported by bootstrap values the level of P increase to 0.35. That says to some sequences does not have sufficient variation or have a lot of variation to defines the *p*. The combined data in a single analysis could provide the total evidence to resolve better the phylogeny of the group.

This combined data set produced an alignment of 2208 positions. No internal in-dels were retained. The analyzed data set included 807 invariant, 1401 variable sites and 754 parsimony informative positions. Among-site rate variation, also common in protein-encoding mitochondrial genomes (Wolstenholme, 1992) was evident for both genes as the vast majority of variation occurred at third codon positions (49.1%) followed by

positions 1 (28.9%) and 2 (22%), respectively. To check the possibility that these genes were saturated at the considered phylogenetic level, we plotted uncorrected *p* distances against the number of substitutions in third codon positions for all species pairs. Visual inspection of the plots revealed a direct relationship between substitutions with the genetic distance, evidence of the absence of saturation (Fig. 1).

The maximum pairwise uncorrected *p* distance among all ingroup species was 0.224 (between *C. gracilis* and *C. elegans*), and the minimum was 0.015 (between *C. limpidus* and *C. infamatus*). The average pairwise divergence value within the genus and with outgroups were 0.113 ± 0.006 and 0.180 ± 0.006 respectively all of these were consistent with the information obtained with the individual *loci*.

Phylogenetic analyses

The combination of diverse analytical approaches, each with a different underlying philosophy, may be particularly useful for testing the robustness of the phylogenetic signal recovered from the data. Two of the groups obtained in the tree (Fig. 2) shown a good statistical support in all the methods (MP, ML and BI) used.

Analyses of the individual loci

In order to assess the individual contribution of each gene in the reconstruction of the phylogenetic tree was observed that despite COI is larger than COII, variability among genes and codon positions based on *Centruroides* species is lower (Table 2).

For COI, ModelTest choused a TrN+G substitution model in AIC with gamma = 0.4201. Maximum likelihood analysis of the sequences with the ModelTest parameters and QP branch support showed that some terminal branches maintained the same relationships as the MP tree in the combined hypothesis. This protein-encoding gene

showed excellent performance in resolving relationships at the level of species and species groups among several taxa (Caterino and Sperling, 1999).

In COII, ModelTest choused a GTR+G substitution model in AIC with alpha = 0.5708. Maximum likelihood analysis of the sequences with the ModelTest parameters and QP branch support showed the division of the two clades: A and B (Fig. 2), but the internal relationships are not well resolved, in comparison with the tree presented with combined data in the MP hypothesis.

When the ML trees obtained with COI and COII genes were compared, just few clades were congruent. In this tree (Fig. 3) we observed which clades each gene supported. At a protein level, aminoacid variation is distributed heterogeneously, both within and between genes. Structural features of the proteins are not always reliable predictors of variation. On the other hand, trees obtained by the protein sequence of each gene (not shown), are not sufficient to resolve the phylogeny of the species in this study.

Analyses of the combined genes

Maximum Parsimony analysis

Parsimony analysis of the sequences using PAUP* resulted in two MP trees with 3650 steps with a Consistency Index (CI)= 0.5912 and a Retention Index (RI)= 0.4720. A majority-rule consensus tree showed high values. One of the two MP trees was selected. The bootstrap values obtained showed high branch support (Fig. 2).

These analyses presented *C. gracilis*, *C. nigrovariatus*, *C. noxius*, *C. sculpturatus* and *C. vittatus* as basal species in the tree. The rest of the species were divided in two principal clades, supported by bootstrap values higher than 50%. Group A was further divided in two subclades, the first one contained ((*C. baergi*, *C. margaritatus*), (*C. fulvipes*, (*C. pallidiceps*, *C. ochraceus*))). This subclade was present in all the analyses

performed. The second subclade contains the species ((*C. limpidus*, *C. nigrimanus*), (*C. infamatus*, *C. nigrescens*)). The relationships between these two subclades were supported by bootstrap values higher than 50%. Group B consisted in two sublades containing (*C. suffusus*, (*C. thorelli*, *C. elegans*)) related with (*C. flavopictus*, *C. exilicauda*). All these branches were well supported and were present in all performed trees.

ModelTest and Maximum Likelihood analyses

In the ML analysis, ModelTest identified the optimal evolutionary model as GTR + G. The parameters of the model were as follows: base frecuencies, A = 0.2564, C = 0.1329, G = 0.2072, T = 0.4035; substitution rate matrix, A-C = 0.6125, A-G = 5.5004, A-T = 0.9628, C-G = 0.9269, C-T = 2.3595, G-T = 1.0000; proportion of invariable sites = 0; gamma shape parameter = 0.4507. Using these parameters we recovered a single unconstrained tree (-lnL = 18179.1875) (not shown). ML analysis supports some but not all the relationships proposed in the MP tree, the statistics Quartet-Puzzle (Strimmer and Haese, 1997) values are shown in the tree (Fig. 2). In a combined analysis, these sequences sustain a nearly fully resolved topology within species group, though higher level relationships among species groups require additional study.

Bayesian Inference

The analysis performed by Bayesian methods showed some variations in the topology. Species belonging to the second subgroup of the group A (Fig. 2) were not well solved and showed polytomies in different parts of the topology. Other difference were that the

species presented as basal group in the MP tree (C. vitattus, C. sculpturatus and C. noxius) were located as sister group of the group B.

Centruroides relationships and comparison with morphologic data

The phylogenetic relationships of *Centruroides* based on adult morphological characters and the present molecular characters hypothesis differ considerably. The first analysis uses some morphological and morphometric characters and results in a division of three principal groups in *Centruroides*: the gracilis, thorelli and the stripes group (Dávila, *et al*; *in press*). As we can see in the tree obtained with the molecular data, this division is not supported because it only shows two groups (Fig. 2). However, group A mostly contains the only two species that belong to the thorelli's group and the majority of species from the stripes group, and in group B with exception of *C. gracilis* contains all the species within the gracilis group.

When we map the morphologic and morphometric characters in the molecular tree obtained, no one of the characters more used by the taxonomist share the division obtained with the molecular tree. The conflict between morphologic and molecular data is not a new thing, we can attribute the differences to some properties of the morphological characters, in first place the small number of these, comparing with the number of characters obtained in the molecular data and in second place the low bootstrap support obtained in some branches of the morphological tree.

Conclusions

This study clearly demonstrates the utility of the COI and COII genes sequences for the study of species group-level relationships in *Centruroides*. The analysis shows the evolutionary relationships among these species that can be useful for the identification and classification in posterior work. The present study supports the hypotheses about natural groups and their relationships in *Centruroides*. This constitutes one of the first molecular analysis of *Centruroides* and entails a rather broad representation of groups; future studies should probably focus on a more thorough taxon sampling of the group and the inclusion of other molecular markers like other mitochondrial genes (*i. e. nad1*, *cytb*) that could be obtained with the complete mitochondrial genomes of scorpion sequenced.

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Table 1 Species used in this study

Family Buthidae Genus *Centruroides*	Collection locality	Collector	GenBank Accesion no.
C. baergi	So. Pedro Huamelula, Oaxaca	R. Cancino	cox1 AY995823, cox2 AY995841
C. elegans	Chamela, Jalisco	S. Dávila, E. Gonzalez	cox1 AY995824, cox2 AY995842
C. exilicauda	Ensenada, Baja California	A. Licea	cox1 AY995833, cox2 AY995851
C. flavopictus	San Andres Tuxtla, Veracruz	S. Dávila, E. Gonzalez	cox1 AY995825, cox2 AY995843
C. fulvipes	Mazunte, Oaxaca	S. Dávila, E. Gonzalez	cox1 AY995834, cox2 AY995852
C.gracilis	Monte Obscuro, Veracruz	S. Dávila, E. Gonzalez	cox1 AY995830, cox2 AY995848
C. infamatus	Ajijic, Chapala, Jalisco	E. M. Frias	cox1 AY995826, cox2 AY995844
C. limpidus	Cuernavaca, Morelos	C. Balderas	AY803353
C. margaritatus	La Gloria, Chiapas	S. Dávila, E. Gonzalez	cox1 AY995827, cox2 AY995845
C. nigrescens	Acapulco, Guerrero	S. Dávila	cox1 AY995828, cox2 AY995846
C. nigrimanus	La Gloria, Chiapas	S. Dávila, E. Gonzalez	cox1 AY995838, cox2 AY995856
C. nigrovariatus	Mitla, Santa Ana, Oaxaca	S. Dávila	cox1 AY995837, cox2 AY995855
C. noxius	Tepic, Nayarit		cox1 AY995829, cox2 AY995847
C. ochraceus	Kalakmul, campeche	S. Dávila, E. Gonzalez	cox1AY995840, cox2 AY995858
C. pallidiceps	Querétaro, Querétaro	C. Equigua	cox1AY995836, cox2 AY995854
C. sculpturatus	Arizona, USA	A. Licea	cox1 AY995831, cox2 AY995849
C. suffusus	Cerro de los Remedios, Durango, Durango	E. M. Frias	cox1 AY995839, cox2 AY995857
C. thorelli	La Trinitaria, Chiapas	A. Ramirez	cox1 AY995832, cox2 AY995850
C. vittatus	El Nogal, Aguascalientes, N.L (SONORA)	E. Gonzalez	cox1 AY995835, cox2 AY995853
Mesobuthus gibbosus			AJ716204
Hubronattus oregonensis			NC 005942
Limulus polyphemus			NC 003057

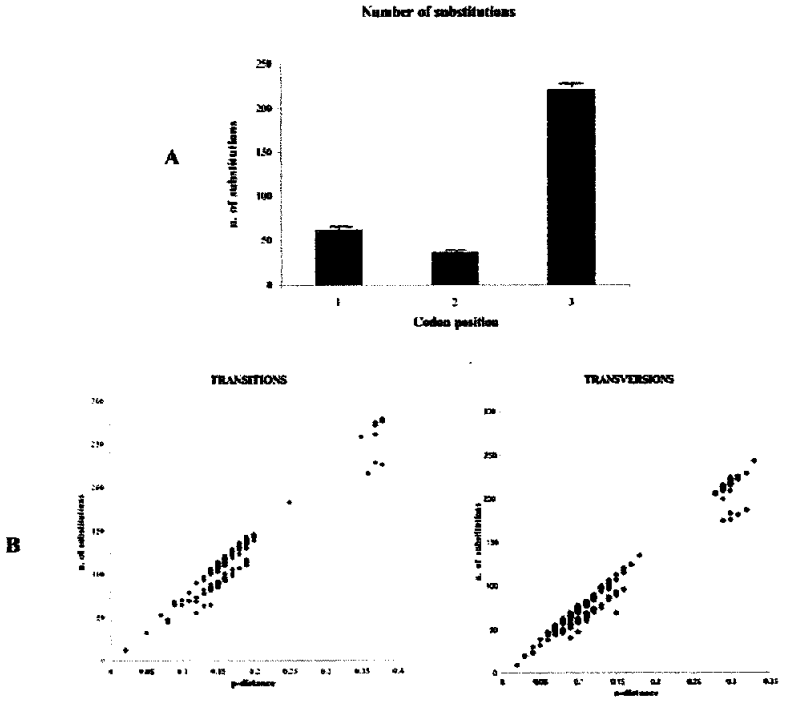
Table 2 Distribution of Variability among Genes and Codon Positions Based on *Centruroides* Species

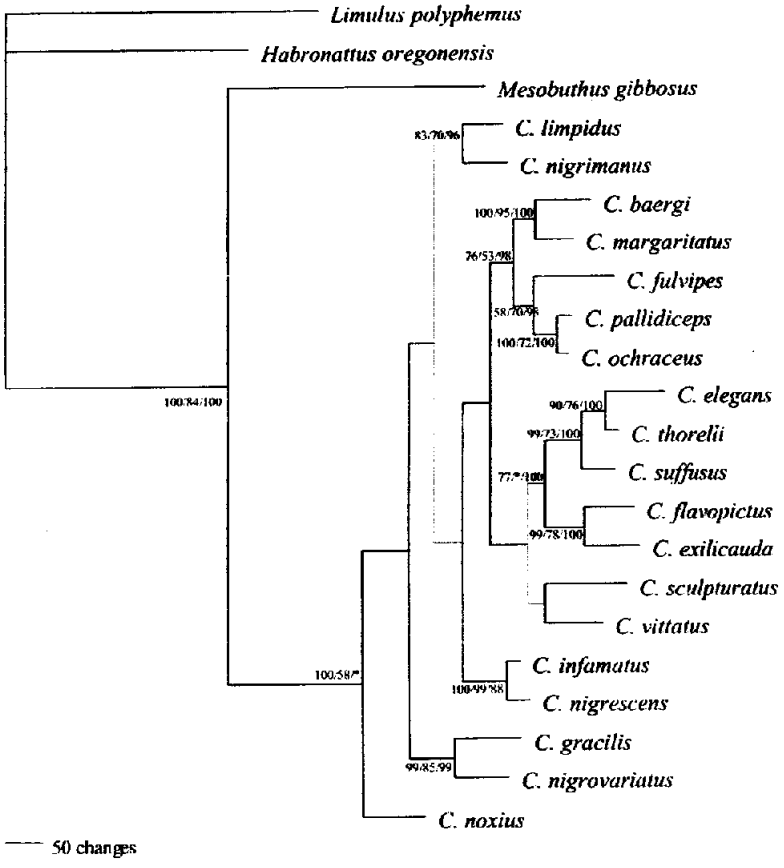
	COI	COII	All sequence
Total sites	1530	678	2208
Total variable sites /percent of total sites	534/34.9%	300/44.3%	834/37.8%
1st position variable/% of 1st positions	102/19.1%	72/24%	174/20.9%
2nd position variable/% of 2nd positions	77/14.5%	60/20%	137/16.5%
3rd position variable/% of 3rd positions	355/66.5%	168/56%	523/62.7%
Aminoacids variable/% of aminoacids	132/25.9%	88/39%	220/28.9%

Legends and Figures

Fig 1 A. Means (± SD) number of substitutions in pairwaise comparisons among *Centruroides* and outgroups species for first, second and third codon positions. **B.** Number of transitions and transversions in pairwise comparisons are plotted against *p* distances in the third positions of combined data.

Fig 2. Phylogeny of *Centruroides* as inferred from MP analysis with COI-COII sequences. Trees were rooted with *L. polyphemus*, *H. oregonensis* and *M. gibbosus*. Branches are drawn in proportion to the estimated number of changes. Numbers indicate bootstrap support percentages by MP/QP (Quartet Puzzling) values of ML/values of Mr. Bayes. * is shown when the values are ≤ 50%.


Number of substitutions
A
n. of substitutions
250
200
150
100
50
0
1
2
3
Codon position
B
TRANSITIONS
TRANSVERSIONS
n. of substitutions
p-distance


Limulus polyphemus
Habronattus oregonensis
Mesobuthus gibbosus
C. limpidus
C. nigrimanus
C. baergi
C. margaritatus
C. fulvipes
C. pallidiceps
C. ochraceus
C. elegans
C. thorelii
C. suffusus
C. flavopictus
C. exilicauda
C. sculpturatus
C. vittatus
C. infamatus
C. nigrescens
C. gracilis
C. nigrovariatus
C. noxius
83/70/96
100/95/100
76/53/98
58/70/98
100/72/100
100/84/100
90/76/100
99/73/100
99/78/100
100/58/*
100/99/88
99/85/99
50 changes

CAPITULO V

Resumen

Una de las controversias existentes dentro del género *Centruroides*, es que no se ha aclarado si *C. exilicauda* y *C. sculpturatus* son especies sinónimas o no.

Algunas observaciones morfológicas han sido tomadas como evidencia para considerar a estos alacranes como especies separadas. Pero algunos autores consideran que estos datos no son suficientes. Por fortuna en la actualidad se cuenta con técnicas bioquímicas y de biología molecular, que permiten corroborar la definición de especies obtenida por características morfológicas clásicas. En este artículo se utilizan estas técnicas, evaluando la toxicidad de cada uno de los venenos obtenidos de los dos alacranes, al realizar separaciones cromatográficas, y secuenciar tanto toxinas como marcadores moleculares mitocondriales para su análisis filogenético. Las diferencias que se muestran son claras, por lo que se concluye que *C. exilicauda* encontrada en Baja California, México, es una especie diferente a *C. sculpturatus*, cuya distribución se presenta en el estado de Arizona en E.U. y Sonora, México.

Este trabajo es un ejemplo de la utilidad de los marcadores mitocondriales, en la clasificación y determinación de la posición filogenética entre las especies.

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Biochemical, genetic and physiological characterization of venom components from two species of scorpions: *Centruroides exilicauda* Wood and *Centruroides sculpturatus* Ewing

Norma A. Valdez-Cruz [a], Sonia Dávila [b], Alexei Licea [c], Miguel Corona [a], Fernando Z. Zamudio [a], Jesús García-Valdes [a], Leslie Boyer [d], Lourival D. Possani [a,*]

[a] Department of Molecular Medicine and Bioprocesses, Institute of Biotechnology, National Autonomous University of Mexico, Avenida Universidad 2001, Apartado Postal 510-3, Cuernavaca 62240, Mexico
[b] Department of Genetic Evolution, Institute of Ecology, National Autonomous University of Mexico, Ciudad Universitaria 04510, Mexico DF, Mexico
[c] Department of Aquaculture, Marine Biotechnology, Centro de Investigación Científica y de Educación de Ensenada, Km 107, Carretera Tijuana-Ensenada, Ensenada BC, Mexico
[d] Arizona Poison and Drug Information Center, University of Arizona Health Sciences Center 1501 N. Campbell avenue, Tucson, AZ 85724, USA

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Abstract

Current literature concerning the taxonomic names of two possibly distinct species of scorpions from the genus *Centruroides* (*sculpturatus* and/or *exilicauda*) is controversial. This communication reports the results of biochemical, genetic and electrophysiological experiments conducted with *C. exilicauda* Wood of Baja California (Mexico) and *C. sculpturatus* Ewing of Arizona (USA). The chromatographic profile fractionation of the soluble venom from both species of scorpions is different. The N-terminal amino acid sequence for nine toxins of *C. exilicauda* was determined and compared with those from *C. sculpturatus*. Lethality tests conducted in mice support the idea that *C. exilicauda* venom should be expected to be medically less important than *C. sculpturatus*. Thirteen genes from the venomous glands of the scorpion *C. exilicauda* were obtained and compared with previously published sequences from genes of the species *C. sculpturatus*. Genes coding for cytochrome oxidase I and II of both species were also sequenced. A phylogenetic tree was generated with this information showing important differences between them. Additionally, the results of electrophysiological assays conducted with the venom from both species on the Ca^{2+}-dependent K^+-channels, showed significant differences. These results strongly support the conclusion that *C. exilicauda* and *C. sculpturatus* are in fact two distinct species of scorpions.

Keywords: *C. exilicauda*; *C. sculpturatus*; Cytochrome oxidase; Mitochondrial gene; Scorpion toxin; K^+-channel

1. Introduction

Scorpions of the family Buthidae comprises approximately 529 species, divided into 73 genera, from which only five are dangerous to humans, but are worldwide spread [1,2]. One of them, the genus *Centruroides*, is distributed from the southern part of USA to Mexico and Central America. From the Baja California area of Mexico, four families, 11 genera, 61 species and 12 subspecies were described [3], which makes it one of the most diverse geographical areas of the world concerning scorpion diversity. Among the first reports is that by Wood [4], who described eight species of scorpions, including two from United States of North America and six from Mexico, state of Baja California. Due to the fact that some of these species are medically important, it was mandatory to clarify species differences and to identify molecular markers and/or structural and functional aspects of their venoms. In this communication we have focused our attention to two species: *C. exilicauda* collected in Punta Banda, Baja California (Mexico) and to

Abbreviations: *C. Centruroides*; COX I, cytochrome oxidase I; COX II, cytochrome oxidase II; I. *Imperator*; mDNA, mitochondrial DNA; T. Tityus

* Corresponding author. Tel.: +52-77-73-171209; fax: +52-77-73-172388.
E-mail address: possani@ibt.unam.mx (L.D. Possani).

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C. sculpturatus, collected in Tucson, Arizona (USA). They are two relatively abundant species of these areas, which for many years have generated taxonomic disputes and controversies about their medical importance. One of the objectives of this work was to clarify the existing uncertainties on these two apparently synonymous species, as well as, to obtain additional characterization of their venom components and gene molecular markers. The medical problem arising from stings of these arachnids, dangerous to humans in Arizona, but apparently not dangerous in Baja California, was pressing a more rigorous study on this issue. Questions demanding some immediate answers were: Are there two different species? Is one dangerous to humans, the other not dangerous? Are both coexisting in both places?

Here we show biochemical differences by chromatographic separations, bioassays, amino acid sequencing of peptides, gene cloning of toxins and by sequencing enzyme markers of both species. Furthermore, for the first time we show results that support the conclusion that *C. exilicauda* from Baja California should be less dangerous to humans than *C. sculpturatus* from Arizona. The results of our morphological analysis of the two species will be included into another communication (S. Davila, N.A. Valdez-Cruz, L.D. Possani, and D. Pifiero, unpublished).

2. Material and methods

2.1. Scorpions

The *C. sculpturatus* Ewing scorpions were collected in Tucson, Arizona, USA. The *C. exilicauda* scorpions were collected in Punta Banda, Baja California, Mexico. For this study 15 animals of each species were used.

2.2. Separation of C. exilicauda and C. sculpturatus venoms

Venoms of both species of scorpions were obtained in the laboratory by electrical stimulation. The venom extract was dissolved in water, centrifuged 15 min at 10,000 × g and the supernatant was lyophilized. Separation of venom components was performed by high performance liquid chromatography (HPLC), as earlier described for the venom of *Tityus cambridgei* scorpion [5].

2.3. Biological tests

Lethality tests were carried out on female albino mice (CD1 strain) of approximately 20 g body weight. Different amounts of venom from both species (*C. exilicauda* and *C. sculpturatus*) were tested in parallel. Injections were performed intraperitoneally using PBS (phosphate buffered saline, containing 0.15 mM NaCl, 0.1 mM sodium phosphate at pH 7.4) plus different doses with a final volume of 100 μl. The intoxication levels were called "non-toxic", when the animals showed no symptoms of envenoming within 20 h after testing, e.g. showed the same symptoms as the control mice injected with 100 μl of buffer alone (PBS). "Toxic" means that the mice showed symptoms such as: excitability, salivation, dyspnea, diarrhea, temporary paralysis, but recovered within 20 h. "Lethal" means that the mice showed some or all the symptoms of intoxication and died within 20 h after injection. For lethality tests we have used the minimum number of animals required to validate the experimental data, according to the guidelines for animal usage of our Institute (the protocols were approved by the Institutional Committee for Animal Welfare).

2.4. Electrophysiological measurements

A pGEM vector containing the cDNA that code for the gene of the human large conductance calcium and voltage dependent K^+-channel, known as MaxiK (hSlo), was expressed on *Xenopus laevis* oocytes, and used for electrophysiological experiments, as earlier described [6]. Holding potential was –60 mV and test pulses were applied from –60 to +80 in 20 mV steps. Data analysis was performed with pClamp 6 (Axon Instruments) or a custom made software kindly provided by Dr. Ligia Toro (see Acknowledgements), and Excel (Microsoft). Bovine serum albumin (0.001%) was added to the venoms and washing solutions. The perfusing volume of solutions containing venom and that containing the washing solutions were about 10 and 20 times greater than that of the chamber containing the oocytes, respectively. Inhibition was evaluated every 2 min until equilibrium was reached, usually from 8 to 12 min. Values are Mean ± S.E.M.

2.5. Scorpion toxins cDNA cloning

Total RNA was isolated from venomous glands located at the last post-abdominal segment (telson) of five *C. exilicauda* scorpions, by the method of Chirwing et al. [7]. Total RNA (500 ng) was used as template to generate cDNA using the oligonucleotide poliT22NN [8]. For gene amplification the following primers were used: (1) 5'-RATGAAYTCG-TTGTTGATGATCA-3'; (2) 5'-GMAARGGARGGTTATC-3'; (3) 5'-RAAGGASGGTTATCCB-3'; (4) 5'-GMAATTAA-GAAGCGTTACAMTA-3'. The PCR products were cloned as described by Corona et al. [8], for the cDNA genes of *C. sculpturatus*. Sequence analysis was conducted in both strands of DNA.

2.6. Mitochondrial-encoded cytochrome oxidase subunits I and II (COX I, COX II)

The mitochondrial DNA (mtDNA) of both scorpion species was extracted as described by Hall and Smith [9]. The genes coding for the enzymes cytochrome oxidase I and II, abbreviated COX I and COX II, were obtained from this material. The primers used for amplification were similar to those earlier described by the authors [9]. The mitochondrial

DNA was amplified using the primer sense 5'-CTACN-AATCATAAAGAYATTG-3' and the antisense 5'-CATAC-CCAAAGARCCAAAAGG-3', for COX I, whereas for COXII the primers were 5'-GTKTGGGCTCATCATATG-3' and 5'-AACTATGATTTGCTCCAC-3'. PCR conditions for COXI were: 94 °C for 60 s, 52 °C for 90 s, 72 °C for 120 s; repeated 30 cycles. For COX II two rounds of amplification were necessary. The first PCR conditions were: 94 °C for 60 s, 46 °C for 90 s, and 72 °C for 120 s, for 30 cycles. The second PCR condition was: 94 °C for 60 s, 46 °C for 90 s and 72 °C for 120 s, for 30 cycles.

2.7. DNA manipulations and sequence analysis

All cloning procedures were performed following the protocols by Sambrook et al. [10]. Nucleotide sequencing was performed using the thermosequenase kit (Amersham Life Science). DNA and protein sequence analyses were performed using DNA strider, BLAST from the Wisconsin Package Version 10.1 (Genetics Computer group (GCG), Madison I, USA), and clustalX from PHYLIP (Phylogeny Interference Package) [11].

2.8. Phylogenetic analysis

The sequences obtained were edited and aligned using ClustalX from PHYLIP package. Trees were constructed by successive clustering of lineages using the neighbour-joining algorithm of Zhang and Nei [12] as implemented in NEIGHBOR program [11]. The unrooted tree diagrams were visualized with Tree-View from Glasgow University (internet reference: http://taxonomy.zoologi.gla.ac.uk/rod/treeview.html/).

3. Results

3.1. Lethality testing

Lethality tests of both venoms were conducted using similar doses reported to be toxic to mammals by Stahnke [13] and Pete et al. [14], when studying the venom of *C. sculpturatus* of Arizona. Table 1 shows the results of application of different amounts of venom to several mice of the strain CD1. At least two mice were used for each doses. The lethal effect for *C. sculpturatus* venom earlier reported was: 1.12 mg/kg mice of a non-specified strain [13] and 0.8 mg/kg of Swiss mice [14]. Our results indicate toxic effects starting at 1.12 mg/kg mouse weight (Table 1), which became lethal in our experimental protocol after injection of 3 mg/kg mouse weight. Doses of 15 mg/kg and higher were not used, in order to avoid unnecessary inflection of pain to experimental animals.

Table 1
Mouse lethality tests for both venoms

Venom dose (mg/kg)	*C. exilicauda*	*C. sculpturatus*
1.12	Non-toxic	Toxic
2.24	Non-toxic	Toxic
3	Non-toxic	Lethal
5	Non-toxic	Lethal
10	Non-toxic	Lethal
15	Toxic	Not tested
25	Lethal	Not tested
50	Lethal	Not tested

Two mice were used for each experiment. The venom of each scorpion was dissolved in 0.1 ml of PBS buffer pH 7.4 and injected intraperitoneally, into adult 20 g albino mice (strain CD-1).

The results of the lethality test using the venom from *C. exilicauda* from Baja California (the species taken for synonymy), are significantly different. The animals injected with this venom do not show any symptoms of intoxication up to the doses of 15 mg/kg mouse weight, and the venom is deadly when using up to 10 times more venom per animal (25 mg/kg), than that of *C. sculpturatus* species (Table 1). In other words, our results are a clear indication of the differences existing at the level of venom toxicity of the species under study.

3.2. Chromatographic profiles of both venoms

The soluble venom from *C. exilicauda* and *C. sculpturatus* scorpions were separated by HPLC in similar conditions. Superimposition of the chromatographic profiles obtained showed the differences (Fig. 1). For these experiments 1.0 mg of venom (mixture obtained from at least 10 scorpions) was applied independently on the column and run with the same buffer gradient for the same time. Most of the protein components, as indicated by the absorbance at 230 nm are eluted with a different retention time. The most prominent differences are in the range of 30–40 min, where most of the known Na^+-channel specific toxins from different *Centruroides* species elute from the C18 reverse-phase column used [15–17].

3.3. C. exilicauda and C. sculpturatus Na^+-channel toxins: purification and partial amino acid sequencing

We have isolated nine major components from the venom of *C. exilicauda* and determined their N-terminal amino acid sequence in order to compare them with those already known from *C. sculpturatus* [8]. Judging by sequence similarities they all belong to the well-known group of the Na^+-channel specific toxins. Two different protocols were used. The first was the direct separation of soluble venom by HPLC. An example of the chromatographic profile obtained with the venom of *C. exilicauda* is shown in dash-lines (main picture of Fig. 1). Four major components indicated by numbers 1–4 were recovered and re-purified as indicated by the upper insets of Fig. 1. A second protocol used a Sephadex G-50 gel filtration chromatography as first step, similar to a procedure well documented earlier for venoms of the genus *Centruroides* [16,17]. Sub-fraction II, which is the one containing the Na^+-channel specific peptides, was further separated by a

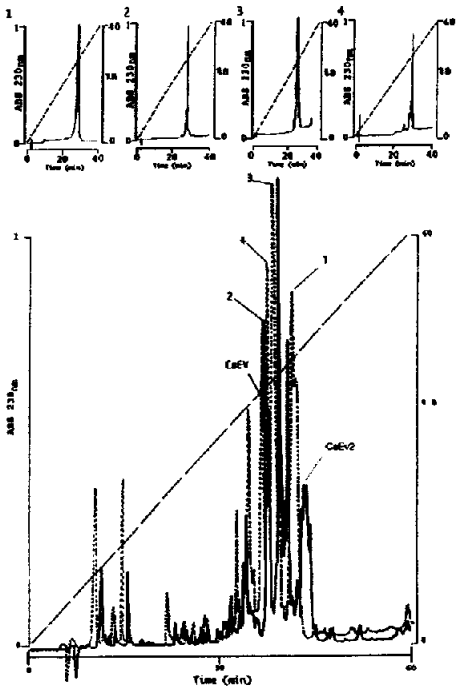

Fig. 1. HPLC separation of soluble venom from *C. exilicauda* (dotted line) and *C. sculpturatus* (continuous line). Soluble venom from each scorpion (1 mg protein) was independently applied to a C18 semi-preparative reverse-phase HPLC column, and eluted with a linear gradient of 100% solution A (0.12% trifluoroacetic acid (TFA) in water) to 60% solution B (0.10% TFA in acetonitrile), run for 60 min. Components labelled 1–4 show elution time for peptides Cex 1–Cex4. These fractions were further separated (see insets labelled 1–4) on a C18 analytical reverse-phase HPLC column using a linear gradient of 100% solution A to 40% solution B, during 40 min. The components marked as CsEv.5 and CsEv.2 correspond to the toxins previously reported [23,44].

C18 reverse-phase column using a linear gradient from 100% solution A to 60% solution B, run for 60 min. Several different peptides were obtained by this means [18], in amounts and purity enough to warrant sequence analysis. Nine peptides were analyzed: numbers 1–4 described here (Fig. 1), and peptides numbered 5–9 obtained by Valdez-Cruz [18]. The peptides were labelled 1–9 preceded by C.e. (abbreviation from *C. exilicauda*). The N-terminal amino acid sequences found were: KEGYLVSKSTGCKYECFWLGKNEGCD for C.e.1; REGYKVNLHNGCKYNCYKXE for C.e.2; KDGYPLASNGCXFGCSGCXEXN for C.e.3; KDGYVSNKTG for C.e.4; KEGYMVNKSTGCXN for C.e.5; KDGYPVDSKGL for C.e.6; KEGYLVXKST for C.e.7; KDGYLVXKXTG for C.e.8; KKDGYPVDSGN for C.e.9, where X means a non-identified residue. Lack of material hampered the determination of the full amino acid sequence of these peptides (work still in progress). The N-terminal and molecular weight (7,136 Da) of the sequence Cex1 corresponded to the Cex1 clone (see below). The N-terminal amino acid sequences obtained provided enough information to conclude that these toxins are different from those published for the species *C. sculpturatus*. Similarly, in order to confirm that the venom from *C. sculpturatus* contained peptides with amino acid sequences of the appropriate

species, we separated (continuous line in Fig. 1), and sequenced two peptides (labelled CsEv2 and CsEV, randomly chosen), which in fact did correspond to the expected known peptides of the species *C. sculpturatus*.

The N-terminal amino acid sequence of the peptide eluted at 33.97 min is compatible with the known sequence of clones of Csv1 [8]. The peptide eluted at 37.74 min, and labelled CsEV, was further purified, reduced, alkylated and sequenced up to the residue 46, giving an identical amino acid sequence to that of toxin CsEV earlier reported by Meves et al. [19]. The major peptide, eluted at 39.20 min, when collected and rechromatographed on a C18 analytical column showed to contain at least four peptides. One of them had an amino acid sequence identical to that of CsEv2, already known to be present in the venom of *C. sculpturatus*. The most important conclusions from these results are: (1) the amino acid sequence obtained from nine peptides purified from *C. exilicauda* showed that the sequences are not identical to those of *C. sculpturatus*. However, as expected, some closely related similarities exist between C.e.7 and CsEv1d, C.e.8 and CsEL and C.e.9 and CsEV; (2) the peptides isolated from the venom of *C. sculpturatus* in our conditions do correspond to known amino acid sequences of peptides or to the deduced clones obtained for this species [8]; (3) finally, some of the peptidic sequences found for *C. exilicauda*, such as C.e.7., and C.e.8 very likely correspond to the peptides coded by genes Cex1 (or Cex2) and Cex5, respectively, as it will be discussed later. Thus, our biochemical data support the fact that although some similarities exist in terms of primary structure analysis of several peptides purified from both species, it is clear that they present many differences in their amino acid sequences.

3.4. Electrophysiological assays of both venoms

Fig. 2 shows the effect of soluble venoms from *C. exilicauda* and *C. sculpturatus* on the human large conductance calcium and voltage dependent K^+-channel (MaxiK), using the experimental conditions described above. Several test pulses were applied, every 30 s, in order to evaluate the current stability. Only oocytes showing a minimum run down were chosen for experimental assays. Fig. 2a shows the control currents through MaxiK channels under whole cell recording. The addition of *C. exilicauda* whole venom (22 μg/μl) blocks outward currents at positive voltages, including 0 mV (Fig. 2b). Fig. 2e shows the mean blockade values at 40, 60 and 80 mV after 8 min equilibration. At +80 mV, the blocking effect of *C. exilicauda* venom is about 90 ± 5% ($n = 4$). Recovery of the currents was incomplete (data not shown). However, *C. sculpturatus* venom (25 μg/μl) only exhibited a marginal current decrease as compared to the control currents at any voltage values (Fig. 2c and d). Again, Fig. 2e shows that there is no significant blocking effect of *C. sculpturatus* venom at 40, 60 and 80 mV after 12 min equilibration. Inhibition effect by *C. sculpturatus* venom, at +80 mV, is only (4.8 ± 2.0) percent with ($n = 4$).

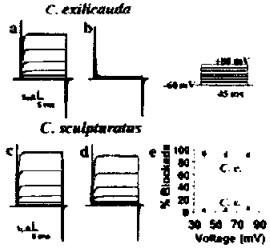

Fig. 2. Blockade of MaxiK$^+$-currents by whole venom of *C. exilicauda* and *C. sculpturatus* scorpions. Macroscopic K$^+$-currents were recorded under whole configuration using two-electrode voltage clamp in ND96 solution. Currents were elicited by depolarizing the membrane for 45 ms, from a holding potential of −60 mV to +80 in 20 mV steps (upper right panel). Pulse protocols were applied at maximum rate. Panel a and c show traces in control conditions, those in b and d in the presence of whole venom from scorpions indicated in the figure. Panel e shows the percentage of blockade of MaxiK currents by *C. exilicauda* venom (labelled C.e., squares), after 8 min of application (22 μg/μl). Similarly, the same panel shows the application of 25 μg/μl of *C. sculpturatus* venom (labelled C.s., triangles). The inhibition of currents by C.e. as shown in b) was not reversible.

3.5. cDNA cloning and sequencing

Using cDNA obtained from mRNA of *C. exilicauda* venomous glands, 50 clones were isolated. The DNA sequence determined for these clones showed that at least thirteen of them would code for peptides having the size and amino acid sequences similar to other known scorpion toxins [20]. The results found are shown in Fig. 3. The FASTA program was used to compare the sequences of Fig. 3 with other scorpion toxins of the genus *Centruroides* (data not shown), and about 80% similarity was found with homologous toxins from *C. sculpturatus* venom [8], but also with toxins from *C. noxius* [21,22]. The sequences of these thirteen peptides are similar to those isolated from *C. sculpturatus* and shown by Meves et al. [19] to be specific for Na^+-channels of frog and chicken [23,24]. However, none of the clones reported here coincided entirely with the 16 genes recently cloned by our group from the venomous glands of *C. sculpturatus* scorpions [8]. Similarly, none of the amino acid sequences obtained for other toxins reported previously [19,23,24] are identical to the peptides coded by the genes now cloned from *C. exilicauda*, or to their N-terminal sequences determined in this work, as previously discussed (see above).

3.6. Phylogenetic analysis of mitochondrial genes

Mitochondrial DNA (mtDNA) provides new features to describe variations between species with high morphological similarities [25,26]. In this work the mitochondrial cytochrome oxidase I (COXI) and cytochrome oxidase II (COXII) were amplified by polymerase chain reaction using

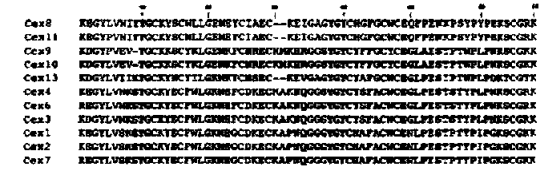

Fig. 4. Amino acid sequence deduced from cDNAs genes cloned from *C. exilicauda*. The first column corresponds to the abbreviated names of the cloned cDNA genes (Cex1–Cex13). They are placed according to similarities aligned by ClustalX (reason why they are not in strictly numerical order). The numbers on top of the amino acid sequence correspond to the position of the residues into the primary sequence deduced. A variable number of gaps (–) were included by the program in order to enhance identity.

purified DNA as template, sequenced and used as molecular marker to differentiate *C. exilicauda* and *C. sculpturatus* species. Fig. 4 shows the amino acid sequence deduced from the mtDNA COX I and COX II nucleotide sequences (GeneBank accession numbers AY648948 and AY648949, respectively) for both species. For comparative purposes, another amino acid sequence of COX I and COX II (S. Dávila, and D. Pinciro, unpublished), obtained from the scorpion *C. limpidus tecomanus* (here abbreviated *C. l. tecomanus*) was included. The orthologe sequences of COX I and COX II of the insect *Apis mellifera* (GeneBank AY114480) [27], were also included for the same purpose. The identity values obtained by using the clustalX program (see Section 2) gave the following results for this comparison: COX I of *C. exilicauda* and *C. l. tecomanus*, 88.0%; *C. exilicauda* and *C. sculpturatus*, 90.4%; *C. sculpturatus* and *C. l. tecomanus*, 87.8%. For COX II, the values were: *C. exilicauda* vs. *C. l. tecomanus* 89.8%; *C. exilicauda* vs. *C. sculpturatus* 89.8%; *C. sculpturatus* vs. *C. l. tecomanus* 90.1%. The comparative identity value for the COX II of *Apis mellifera* is much smaller. For example, only 44.8% was found between *A. mellifera* and *C. sculpturatus*, as expected. Even lower values were obtained comparing with the other species (data not shown). Although the percentages values of identity between *C. exilicauda*, *C. sculpturatus* and *C. l. tecomanus* are relatively high, showing around 10% divergence, they are indeed sufficient to support their classification as independent species as it has been shown for other cases in the literature. For example, see the case of COX I divergence of the milkweed beetles of the genus Tetraopes [28].

Fig. 5 shows a phylogenetic unrooted tree obtained with the nucleotide sequences of COX I and COX II of the same four species, for which the amino acid sequences are shown in Fig. 4. The distances separating each one of the species in Fig. 5 are a graphic representation of the percentage of divergence between each one of them. The left panel was obtained for COX I and the right panel for COX II. Based on these molecular markers, *C. exilicauda* and *C. sculpturatus* are separated by values comparable to those found for *C. l. tecomanus*, definitely described as an independent species. In this tree the divergence values found for the insect markers (*A. mellifera*) are more distant to those of the scorpions (*Centruroides* sp.), as expected (see lengths of horizontal lines).

4. Discussion

Earlier morphological observations were taken as sufficient evidences for taxonomic classification of animal species, including scorpions [4,13,29]. Wood [4] and Ewing [29] have initially classified the scorpions *C. exilicauda* and *C. sculpturatus* as distinct species. However, Williams [3] revised their morphological characteristics and concluded that they were circumstantial and both types of scorpions should be considered as *C. exilicauda*. Fortunately, biochemical and molecular markers have been recently used to corroborate the classical morphological characteristics used for species definition. Some species of scorpions from the genus *Centruroides* are medically important, causing human fatalities [30–32]. Their venom contain a variety of biologically active components such as peptides, enzymes, nucleotides, lipids, mucoproteins and other unknown substances [15,31,33]. The best studied components are polypeptides that recognize ion channels and other receptors in the membranes of excitable and non-excitable cells [20,34–42]. Four different families of toxins have been described, according to the ion channel that they recognize: Na^+-channels [20,34], K^+-channels [35–37], Cl^- channels [38] and Ca^{2+} channels [39–42]. The composition and distribution of these various active components depend on the species under study. Thus, the isolation and characterization of peptides and genes from scorpions should be taken as bonafide data for the novel systematic of scorpion species.

When we first isolated the cDNA genes from *C. sculpturatus* from Tucson, Arizona and submitted our data to the GeneBank in the year 2000, under the name *C. sculpturatus* with the intention to be consistent with previous publications [19,23,24,43,44], on Arizona scorpions, our data were not accepted as belonging to the species *C. sculpturatus*, and were register under the name of *C. exilicauda*. In view of the

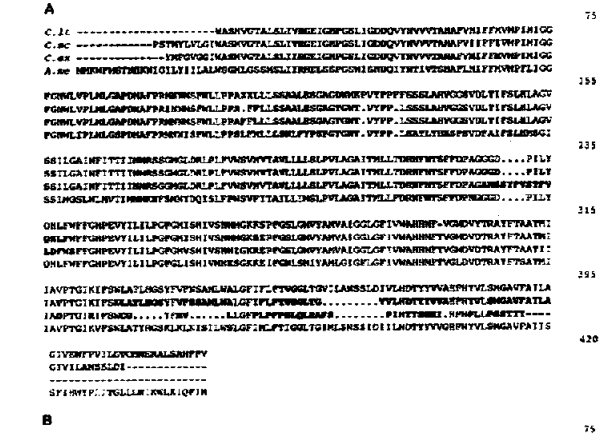

Fig. 4. Amino acid sequence deduced from the genes of COX I and COX II of *C. exilicauda* and *C. sculpturatus*. The sequences obtained from mitochondrial DNA of both species are reported here together with that of *A. mellifera* and *C. limpidus tecomanus*, for comparative purposes. Upper-left column labeled with A.me, C.ex, C.sc, C.li, means sequences of *A. mellifera*, *C. exilicauda*, *C. sculpturatus* and *C. limpidus tecomanus*, respectively for COX I in A and for COX II in B. The numbers on the right column indicate the amino acid position of the last residues in the alignment of *A. mellifera*. The alignments were obtained using ClustalX as described in Section 2. Gaps were introduced by the program in order to enhance identity, and bold amino acids indicate those that are variable residues, when comparing only C.ex with C.sc. The dashed lines at the beginning of the sequences (N-terminal region, positions 1–12) for both COX I (A) and COX II (B) and at the end of the sequence, around position 407 (C-terminal region) were not determined (see A). These undetermined stretches of sequence were due to the strategy used for sequence oligonucleotides used for PCR's.

results presented here and those earlier published by Corona et al. [8], the information presently available in the GeneBank corresponding to cloned cDNA genes of *C. exilicauda* must be revised. It is clear from the Section 3, that biochemical, genetic and evolutionary differences between these two species of scorpions justify the original classification of Wood [4] and Ewing [29], as different species.

4.1. Lethality testing

The results showed that the toxicity of the venom from *C. sculpturatus* is not significantly different from that reported by Stahnke [13] and Pece et al. [14]. Our data compare quite well with those obtained with *C. l. limpidus*, another scorpion dangerous to humans [16]. These authors found variation on the value of LD_{50} (50% lethal doses) for different strains of mice. Depending on the strain of mice the LD50 can vary from 0.61 (strain SSA white) to 3.31 mg/kg (BALB/k, white). Most of the other strains tested by Alagon et al. [16] resulted in LD_{50} close to 1–2 mg/kg mouse weight. It is also important to note that our data do not reflect exact determinations of LD_{50} (again, to avoid usage of many animals) but is a good indication of the toxic potency of both venoms. The results of the lethality tests using the venom from *C. exilicauda* from Baja California are significantly different. Furthermore, these data confirm medical observa-

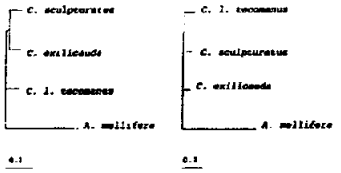

Fig. 5. Unrooted phylogenetic tree of COX I and COX II from *C. exilicauda* and *C. sculpturatus*. Left panel: Multiple alignment of nucleotidic sequences of COX I from *C. sculpturatus*, *C. exilicauda*, *C. l. tecomanus* and *A. millefora*. This phylogram shows the distances of divergence for the four sequences, where the scale (0.1) means the probability of one change occurrence in 1000 nucleotides. Right panel: The same results for COX II.

tions of human accidents occurred with *C. sculpturatus* of Arizona vs. *C. exilicauda* of Baja California. The first one has a definitely medical importance [28,45], confirmed by unpublished observations by one of the co-authors of this work (L.B.), from the Poison Center in Arizona, whereas the second one from Baja California represents no risk for human (personal experience of the first author of this communication). This fact was additionally confirmed by reviewing the medical records, at the National Institute of Public Health, in Cuernavaca, Mexico. This Institute has a complete statistical record of scorpion intoxication cases in the country (Mexico) and there is no record of fatal accidents in Baja California, due to scorpions (data not shown). Thus, the venom from *C. sculpturatus* is dangerous to humans, but the venom of *C. exilicauda* is not.

4.2. Electrophysiological characterization

Using MaxiK channels, expressed in oocytes, it was possible to show the existence of marked physiological differences, when both venoms were comparatively assayed, in this specific ion-channel. It is worth mentioning that blockade of MaxiK channel by some of these toxins do not necessarily causes toxicity symptoms on experimental animals, assayed in vivo. Charybdotoxin was the first scorpion toxin specific for MaxiK described [21]. Injection of charybdotoxin in experimental mice does not cause observable intoxication symptoms (C. Miller, personal communication). This result does not contradict our data. Although *C. exilicauda* lethality tests (see Table 1) show to be less toxic than that of *C. sculpturatus*, it is the one that have an effective component for the blockade of the MaxiK (Fig. 2). The clinical manifestations found when injecting venom from *C. sculpturatus* suggest that the toxic effect seeing in vivo, is probably due to peptides that affect other ion-channels, as it is certainly known for the Na^+-channel specific toxins [20].

4.3. cDNA cloning

If we compare the amino acid sequence of toxins directly determined by Edman degradation with those deduced from the cDNA clones, it might be possible to find certain degree of identity, even between different species of scorpions. When the full amino acid sequences of all these peptides become available it would be not surprising if some peptides from both species turn out to be identical. This was found for the case of butantoxin, a K^+-channel specific toxin present in identical form in the venom of four different species of scorpions of the genus *Tityus*: *T. serrulatus*, *T. bahiensis*, *T. stigmurus* [46] and *T. trivittatus* [47]. The diversity and variations of the cDNA gene sequences found when comparing *C. exilicauda* from Baja California with *C. sculpturatus* from Arizona, are a reasonable indication that they belong to two distinct species of scorpions.

4.4. Phylogenetic analysis of peptide toxins and cytochrome oxidases

A phylogenetic unrooted tree, using the neighbour-joining method [12] was generated with the amino acid sequences coded by all the cDNA genes cloned from *C. exilicauda*, whose sequences corresponded to peptides similar to the Na^+-channel specific toxins (Fig. 6, left). Similarly, for comparative purposes, the deduced amino acid sequences from 16 genes cloned from the scorpion *C. sculpturatus* [8] were analysed together with the 13 peptides of *C. exilicauda* (Fig. 6, right).

The left panel in Fig. 6 shows that the peptides obtained from *C. exilicauda* have important similarities, although situated in at least two separated branches. When this analysis is performed with all the genes cloned from both species (right panel), it is clear that most of *C. exilicauda* sequences are clustered around the middle of the graphic, whereas in the upper and lower extremes are those from *C. sculpturatus*, except for Cex12 and Cex13 that commonly show short

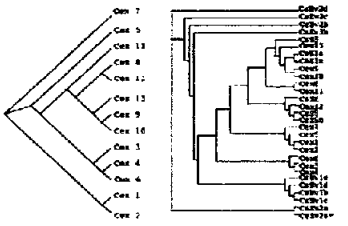

Fig. 6. Unrooted phylogenetic three of toxins from *C. exilicauda* and *C. sculpturatus*. Left panel: Multiple alignments of 13 amino acid sequences from *C. exilicauda* were used to calculate a matrix with genetic distances (slanted cladogram), using the neighbour-joining algorithm mentioned in Section 2. The abbreviations (Cex with numbers) correspond to the 13 cloned cDNAs. Right panel: Multiple alignments of 29 amino acid sequences, of which 13 are from *C. exilicauda* (this work) and 16 from *C. sculpturatus* [8], using the same software as for the left picture. The results are shown in a rectangular cladogram. Abbreviations on the right column correspond either to *C. exilicauda* (Cex) or to *C. sculpturatus* (abbreviated CsE, plus a letter or number according to Corona et al. [8].

phylogenetic distances. These results point out to the same direction as those previously discussed above, when comparing the N-terminal amino acid sequences of the peptides purified from both species. There is no doubt that both species have similar genes coding for similar peptides, although not identical. The genes from a given species are more related among themselves than to the genes of the other species, when simultaneously compared. These results again, suggest that we are dealing here with two distinct species of scorpions.

5. Conclusion

This communication reports data on mice toxicity tests, chromatographic separations, cloned genes and electrophysiological characteristics of venom from two scorpions, whose taxonomic classification biochemical, genetic and electrophysiolgical data are under discussion. One of the scorpions is endemic on the area of the California Peninsula, whereas the other is from the Continental region. Concerning this subject, recent data published by Riddle et al. [48], on a phylogeographic population structure across a suite of 12 mammalian, avian, amphibian, and reptilian species and subspecies of the Baja California desert area, give evidence that this Peninsular Desert can no longer be considered a subset of the Sonoran Desert. Thus, the presence of *C. exilicauda* in Baja California and that of *C. sculpturatus* in Arizona (and Sonoran desert), are also not consistent with the proposal that both species are synonyms.

For these reason we can conclude that *C. exilicauda* found in Baja California is a bonafide different species than that of *C. sculpturatus* found in the Arizona or Sonoran geographical area. Similar studies are expected to be performed for clarification of the existence of a monophyletic lineage among the *C. exilicauda* scorpions found along the Peninsula (North area of Baja California vs. South Baja California).

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CAPITULO VI

DISCUSIÓN GENERAL

Durante el desarrollo de esta tesis, se abordaron distintos problemas relacionados con la filogenia y evolución de los alacranes a distintos niveles jerárquicos. En primer lugar haciendo una contribución al acervo genético sobre este grupo tan controversial, se obtuvo la secuencia completa del DNA de la mitocondria de *C. limpidus*, lo que permitió determinar las diferencias y similitudes primero entre los dos alacranes y después con las demás especies dentro de Chelicerata, las cuales incluyen a la especie basal del xiphosuro *L. polyphemus*; algunas especies del orden Acari, que es el mejor representado, y algunas especies de Aranae. Esta contribución además permite realizar pruebas de frecuencias relativas para la comparación de linajes y conocer sus tasas de evolución. Aunque esto representa una pequeña muestra de todos los ordenes que componen a la clase Arachnida, el análisis no apoya que los alacranes sean un grupo basal hermano de los demás ordenes de Arachnida como apoya Weygoldt y Paulus en 1979. Por el contrario los alacranes aparecen como grupo hermano de Aranae, el cual es considerado uno de los grupos que se han derivado más recientemente. Esto apoya la propuesta que algunos autores hicieron, como Lankester en 1881, que hace la división entre Aerobranchia y Lipobranchia y Shavrov en 1966, al considerar a las arañas y alacranes monofiléticos por la presencia de pedipalpos, otra de las propuestas había sido la de considerar a los alacranes relacionados con los Acari en un grupo denominado Latigastra, este grupo propuesto por Petrunkevitch en 1955, no se ve apoyado con el análisis realizado. En los trabajos más recientes (Shultz, 1990 y Wheeler y Hayashi, 1998) en donde se incluyen también datos moleculares, muestran a los alacranes como un grupo derivado más relacionado con los Opiliones, Pseudoscorpiones y Solifugos, debido a la ausencia de representantes de estos grupos no nos es posible descartar estas relaciones. En este trabajo no se utilizaron las secuencias completas del DNA mitocondrial debido a que muchos de estos genes son muy variables, tal es el caso de los tRNA´s y de algunos genes que codifican para proteínas como ATP8, NAD2 y NAD6. Los Xiphosuros representados por la especie *L.*

polyphemus fueron utilizados para el análisis filogenético como grupo externo, ya que es considerado el grupo más ancestral dentro de Chelicerata.

La segunda parte del trabajo incluye el análisis filogenético del género *Centruroides*. Si tomamos en cuenta que la familia Buthidae tuvo su origen en el continente llamado Laurasia, que hasta hace apenas 15 millones de años se unieron Norte y Sudamérica y que este género sólo se encuentra en el continente americano (Nuevo mundo), podemos ubicar a México como el lugar ideal para el estudio de las especies que dieron origen a la diversidad existente dentro de *Centruroides*. México presenta una gran diversidad de alacranes, pero todos los que son considerados tóxicos para los humanos pertenecen al género *Centruroides*.

El anásisis filogenético se llevó a cabo utilizando datos morfológicos y moleculares. Como se muestra en la Figura 4, en donde los grupos obtenidos a partir de los datos morfológicos se distinguen por los colores y se encuentran sobrepuestos a la filogenia obtenida con los datos moleculares. Las diferencias se observan claramente ya que prácticamente ninguno de los grupos descritos con los datos morfológicos se encuentra en un clado definido, el conflicto entre filogenias de datos morfológicos y moleculares no es nuevo (Hillis y Wiens, 2000), sin embargo, ambos resultados nos permiten resaltar algunas de las características más relevantes dentro del grupo. Uno de los caracteres morfológicos más utilizados para diferenciar especies en el campo es la presencia de rayas en la parte dorsal del carapacho y mesosoma. Hoffmann en sus trabajos sobre alacranes mexicanos llegó incluso a clasificarlos en rayados y no rayados. Esta clasificación está apoyada parcialmente en ambos árboles, ya que se muestra una tendencia de los grupos por favorecer la presencia de especies con rayas ó sin rayas. De los otros grupos nombrados de forma convencional tomando en cuenta caracteres morfológicos como Gracilis, Bertholdii y Thorelli (González, 2001), sólo el grupo Gracilis parece formar un clado bien sustentado, con los datos morfológicos, sin embargo con los datos moleculares, las especies que lo componen no forman un grupo monofilético claro. El grupo Bertholdii, no presenta sustento en ninguno de los árboles y como se mencionó en el artículo con el análisis morfológico, parece ser un grupo formado por aquellas especies excluidas de los demás grupos y no formado por caracteres compartidos entre ellas mismas. Finalmente el grupo Thorelli es monofilético en el árbol

con los datos morfológicos, este grupo no fue evaluado con los datos moleculares ya que no fue posible obtener ejemplares debido a su limitada distribución y abundancia. La única especie analizada fue *C. thorelli* que aparece en el segundo clado formado por especies rayadas principalmente, característica que esta especie comparte. Por otro lado, observando la distribución de las especies reportadas como tóxicas para los humanos, se encuentran en conflicto comparando los dos árboles ya que en el morfológico estas especies se encuentran como derivadas y en el molecular como especies basales.

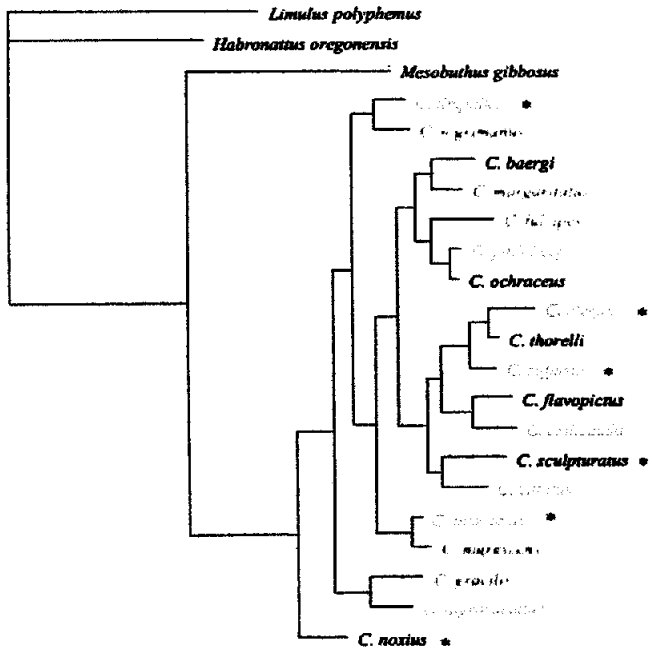

Figura 4 Filogenia obtenida a partir de datos moleculares, cada uno de los colores representa el grupo definido a partir de datos morfológicos. El verde representa a las especies del grupo Gracilis, el azul al grupo Thorelli y el naranja al grupo Rayado. Los asteriscos marcan a las especies tóxicas para el hombre.

En la Figura 4 también podemos observar que la mayor parte de las especies toxicas para el humano recaen en el grupo denominado Rayado, sin embargo, en la filogenia molecular no forman ningún grupo, lo que sugiere que los componentes del veneno tienen un origen muy antiguo ya que la toxicidad hacia el humano se presenta a lo largo del árbol filogenético en especies alejadas que no forman clados.

En la tercera parte del trabajo, se diferencian a partir de distintos tipos de información (moleculares, filogenéticos y de importancia médica) dos especies que hasta el momento eran consideradas como sinónimas. Uno de los puntos interesantes es el de separar a las especies con el 10% de diferencia al comparar los genes COX, esto se ha considerado para algunos escarabajos, sin embargo en el caso de los alacranes podría aumentar esta diferencia ya que son organismos más antiguos aunque menos diversos.

CONCLUSIONES

En conclusión este trabajo hace una aportación importante al estudio filogenético de los alacranes, tanto en su relación y posición evolutiva con respecto a los demás Chelicerata así como del conocimiento sobre las relaciones evolutivas dentro del género *Centruroides*.

La secuencia completa del DNA mitocondrial del alacrán *C. limpidus*, permitió compararla con otras y obtener diversas conclusiones, como: 1) El contenido y el arreglo genómico mitocondrial, está conservado dentro del subphylum Chelicerata; 2) La estructura secundaria de los RNAs de transferencia predicha, la comparten los 3 distintos ordenes de arácnidos secuenciados y esto puede servir para determinar la monofilia de la clase Arachnida, ya que puede ser una característica compartida desde su ancestro común; 3) Con estos datos los alacranes no aparecen como el grupo basal de los demás arácnidos como se ha supuesto en distintos trabajos.

El análisis filogenético realizado con datos tanto morfológicos como moleculares utilizando distintas técnicas de reconstrucción filogenética, apoyan que el género *Centruroides* es monofilético, en ninguno de los árboles obtenidos se muestran taxas sinónimos, por lo que podemos decir que todas los taxas presentados son especies independientes. También se hizo una contribución para diferenciar a dos de las especies más controversiales en donde se concluyó que *Centruroides exilicauda* y *C. sculpturatus* son especies diferentes.

PERSPECTIVAS

A partir de la secuencia del DNA mitocondrial obtenida de *C. limpidus*, será posible seleccionar marcadores moleculares mitocondriales y diseñar oligos específicos para la amplificación de genes o regiones que permitan realizar diversos análisis evolutivos.

Con la incorporación de datos provenientes de los diversos fósiles de alacranes es posible realizar un análisis para determinar el tiempo de divergencia entre los distintos grupos utilizando relojes moleculares.

El análisis mitocondrial de los demás ordenes dentro de Arachnida, en particular los que han sido considerados los más cercanos a Scorpiones como Pseudoscorpiones y Solifugos, es necesario para determinar con certeza la posición de los alacranes dentro de Arachnida.

Dentro del género *Centruroides*, se podrían analizar especies localizadas en el Caribe para determinar las relaciones evolutivas y evaluar su origen.

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