THE FIRST MITOCHONDRIAL DNA PHYLOGENY OF CUBAN BUTHIDAE (SCORPIONES: BUTHOIDEA)

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Abstract: A partial phylogeny of Cuban scorpions of the family Buthidae (four genera, 12 species) is herein presented, based on molecular data (16S rRNA mitochondrial DNA). The following results are discussed: (1) monophyly of the clades (*Rhopalurus* + *Centruroides*) and (*Alayotityus* + *Tityus*) was supported, while *Microtityus* (*Parvabsonus*) always formed a separate clade; (2) monophyly of *Centruroides* was never supported; (3) previous analyses of systematics and morphology-based phylogenies were confirmed.
Key word: Scorpiones, Buthidae, phylogeny, mitocondrial DNA, Cuba.

Primeros datos sobre la filogenia de los Buthidae cubanos (Scorpiones: Buthoidea), basados en ADN mitocondrial.
Resumen: Se presenta una aproximación filogenética parcial a los escorpiones cubanos de la familia Buthidae (cuatro géneros, 12 especies), basada en datos moleculares (ADN mitocondrial 16S ARNr). Se obtuvieron los siguientes resultados principales: (1) la monofilia de los clados (*Rhopalurus* + *Centruroides*) y (*Alayotityus* + *Tityus*) siempre fue confirmada, mientras *Microtityus* (*Parvabsonus*) siempre formó un clado independiente; (2) la monofilia de *Centruroides* no fue confirmada en ningún análisis; (3) se confirmaron los análisis previos de sistemática y filogenia basados en morfología.
Palabras clave: Scorpiones, Buthidae, filogenia, ADN mitocondrial, Cuba.

Introduction

Of the two scorpion families that inhabit Cuba, Buthidae is by far the most diverse. The family is represented by six genera and 35 valid species plus two subspecies. Only five of these species are not endemic, but one of those is indeed autochthonous to Cuba. Thus, Cuba has been of considerable interest to scorpiologists, who published more than 50 papers devoted mostly to Cuban scorpions since 1800. These studies have dealt almost exclusively with systematics and taxonomy, but some minor contributions on ecology, biology and biogeography have also been produced. Most of the works published before 1999 are listed in Armas (1996 [unpublished data]) and Fet & Lowe (2000), with the exception of Navarro & Teruel (1996). Further studies on Cuban scorpions have been published by Teruel (2000a, 2000b, 2001a, 2001b, 2001c, 2002a, 2002b, 2003a, 2003b, 2004a, 2004b, 2006a, 2006b), Teruel & Díaz (2002), Pérez & Teruel (2004) and Teruel & Montano (2005).

Since molecular phylogenetic studies had not been conducted on Cuban Buthidae, the authors decided a few years ago to begin working on this group. Cuban buthids are especially interesting as an exceptionally complex assemblage of genera for which various geographic origins have been suggested (Armas, 1982, 1988, 1996 [unpublished data]; Teruel, 2001b):

1. North and Central American: *Centruroides* Marx, 1890.
2. South American: *Microtityus* Kjellesvig-Waering, 1966 and *Rhopalurus* Thorell, 1876.
3. Endemic: *Alayotityus* Armas, 1973 and *Tityopsis* Armas, 1974.
4. Introduced: *Isometrus* Ehrenberg, 1828.

Especially intriguing is the origin of *Alayotityus* and *Tityopsis*. Both genera are endemic to Cuba and neither share many morphological similarities with other Cuban species. *Tityopsis aliciae* Armas & Martín (1998) was described from a single specimen supposedly collected in southeastern Mexico. A direct study of the holotype (R. Teruel, unpublished data), however, revealed that the taxonomic placement of this strange buthid scorpion in *Tityopsis* was incorrect. Further studies on this matter are currently being conducted by R. Teruel and Oscar F. Francke (in progress).

Preliminary results from some several sequences collected for this study have already been presented at scientific meetings (Teruel *et al.*, 2003; Graham *et al.*, 2004); here we compile and discuss all results obtained to date.

Material and methods

Material. Scorpions were collected in the field and carried live to the laboratory. After being sacrificed by freezing, the pedipalps and legs were cut off with sterilized blades, dried for about two weeks in anhydrous $CuSO_4$ (the remaining trunk was preserved in 94% ethanol), and sent to Marshall University where the DNA extraction was conducted. Apart from the specimens used for DNA extraction, additional specimens with the same collecting data were deposited as vouchers in the personal collection of the first author (RTO).

New DNA sequences used in this analysis were obtained for 12 species of Buthidae from Cuba with the following label data: (1) *Alayotityus delacruzi* Armas, 1973: Santiago de Cuba Province: Santiago de Cuba municipality: Siboney-Juticí Ecological Reserve: Cueva de los Majáes

(type locality); 24 March 2003; UV detection, on cave walls; R. Teruel; (2) *Alayotityus juraguaensis* Armas, 1973: Santiago de Cuba Province: Santiago de Cuba municipality: Playa Juraguá (type locality); 19 August 2001; under rocks; R. Teruel & Y. Pérez; (3) *Alayotityus nanus* Armas, 1973: Santiago de Cuba Province: Santiago de Cuba municipality: road to Piedra del Espejo, El Cobre; 8 August 2000; under rocks; R. Teruel & Y. Pérez; (4) *Alayotityus sierramaestrae* Armas, 1979: Santiago de Cuba Province: Guamá municipality: Río La Mula; 18 June 2003; under rocks; R. Teruel & Y. Pérez; (5) *Centruroides anchorellus* Armas, 1976: Santiago de Cuba Province: Santiago de Cuba municipality: San Juan Botanical Garden; 24 March 2003; under tree bark; R. Teruel & Y. Pérez; (6) *Centruroides baracoae* Armas, 1976: Las Tunas Province: Jobabo municipality: Zabalo; 6-15 March 2003; under tree and palm bark; R. Teruel; (7) *Centruroides gracilis* (Latreille, 1804): Santiago de Cuba Province: Santiago de Cuba municipality: San Juan Botanical Garden; 24 March 2003; under rocks; R. Teruel & Y. Pérez; (8) *Centruroides guanensis* Franganillo, 1930: Las Tunas Province: Jobabo municipality: Zabalo; 6-15 March 2003; UV detection, on the ground; R. Teruel; (9) *Centruroides nigropunctatus* Teruel, 2006: Santiago de Cuba Province: Guamá municipality: Río La Mula; 18 June 2003; R. Teruel & Y. Pérez; (10) *Centruroides robertoi* Armas, 1976: Santiago de Cuba Province: Santiago de Cuba municipality: Siboney-Juticí Ecological Reserve; 24 March 2003; UV detection, on bush branches; R. Teruel; (11) *Microtityus (Parvabsonus) fundorai* Armas, 1974: Santiago de Cuba Province: Santiago de Cuba municipality: San Juan Botanical Garden; 24 March 2003, under stones; R. Teruel & Y. Pérez; (12) *Rhopalurus junceus* (Herbst, 1800): Santiago de Cuba Province: Santiago de Cuba municipality: San Juan Botanical Garden; 24 March 2003; under tree bark; R. Teruel & Y. Pérez.

Methods. *DNA Analysis.* For DNA analyses, total DNA was extracted from dry muscle tissue (usually pedipalp or metasoma) using the Qiagen™ DNeasy extraction kit. Extracted DNA was amplified by the polymerase chain reaction (PCR) in the Perkin Elmer 2400 PCR Thermocycler by using conditions and primers for the mitochondrial LSU (large ribosomal subunit) 16S rRNA as described in Gantenbein *et al.* (1999). The forward primer is a scorpion-specific version of the "universal" primer 16Sbr, or LR-J-12887, while the reverse primer has a scorpion-specific sequence designed by one of the authors (VF). The resulting 400-450 base pair (bp) PCR product was verified on 1% agarose electrophoretic gel and purified with a Qiagen™ purification kit. Automated Sanger dideoxy sequencing of the double-stranded PCR product was obtained at the Molecular Genetics Instrumentation Facility, University of Georgia (Athens, GA), on the ABI 9600 Sequencer.

All 12 new sequences obtained from Cuban Buthidae were deposited in the GenBank nucleotide sequence database (http://www.ncbi.nlm.nih.gov) under the following accession numbers: *Alayotityus delacruzi*, DQ990826; *A. juraguaensis*, DQ990827; *A. nanus*, DQ990828; *A. sierramaestrae*, DQ990829; *Centruroides anchorellus*, DQ990820; *C. baracoae*, DQ990821; *C. gracilis*, DQ990822; *C. guanensis*, DQ990823; *C. nigropunctatus*, DQ990824; *C. robertoi*, DQ990825; *Microtityus fundorai*, DQ990830; *Rhopalurus junceus*, DQ990831. Voucher specimens from

which the abovementioned DNA samples were extracted bear the following catalog codes: *Alayotityus delacruzi*, RTO-DNA0021; *A. juraguaensis*, RTO-DNA0022; *A. nanus*, RTO-DNA0023; *A. sierramaestrae*, RTO-DNA0024; *Centruroides anchorellus*, RTO-DNA0025; *C. baracoae*, RTO-DNA0026; *C. gracilis*, RTO-DNA0027; *C. guanensis*, RTO-DNA0028; *C. nigropunctatus*, RTO-DNA0029; *C. robertoi*, RTO-DNA0030; *Microtityus fundorai*, RTO-DNA0031; *Rhopalurus junceus*, RTO-DNA0032.

Phylogenetic Analysis. The phylogenetic analysis included 23 DNA sequences. For phylogenetic comparisons, in addition to Cuban species, the following nine DNA sequences of Buthidae (published by VF and his coauthors: Gantenbein *et al.*, 2001; Towler *et al.*, 2001; Fet *et al.*, 2003) were taken from GenBank for comparison: (1) *Centruroides balsasensis* Ponce et Francke, 2004: Churumuco, Michoacán, Mexico (AF439758; Towler et al., 2001, as *C. limpidus*); (2) *Centruroides bani* Armas & Marcano Fondeur, 1987: Dominican Republic (AJ288644; Gantenbein *et al.*, 2001); (3) *Centruroides exilicauda* (Wood, 1863): Cabo San Lucas, Baja California, Mexico (AJ288636; Gantenbein *et al.*, 2001); (4) *Centruroides infamatus* (C. L. Koch, 1844): Zumpimito, Michoacán, Mexico (AF439753; Towler *et al.*, 2001); (5) *Centruroides limpidus* (Karsch, 1879), Querétaro, Mexico (AF439758; Towler *et al.*, 2001); (6) *Centruroides sculpturatus* Ewing, 1928: Yuma, Arizona, USA (AJ288640; Gantenbein *et al.*, 2001, as *C. exilicauda*); (7) *Centruroides vittatus* (Say, 1821): Arkansas, USA (AJ288643; Gantenbein *et al.*, 2001); (8) *Grosphus madagascariensis* (Gervais, 1843): Madagascar (AY226168; Fet *et al.*, 2003); (9) *Rhopalurus abudi* Armas & Marcano Fondeur, 1987: Dominican Republic (AY226169; Fet *et al.*, 2003). For phylogenetic comparisons, we also used two unpublished sequences of 16S rDNA available from GenBank: *Tityus bahiensis* (Perty, 1833): Brazil (AY586779; Borges *et al.*, in progress), and *Tityus stigmurus* (Thorell, 1876): Brazil (AY586781; Borges *et al.*, in progress, as *Tityus serrulatus* Lutz & Mello, 1922).

The DNA sequences were aligned using ClustalX 1.81 (Thompson *et al.*, 1997) and by eye. Phylogenetic trees were built using Maximum Parsimony (MP) and Neighbor Joining (NJ) algorithms in PAUP* 4.10b (Swofford, 2000). We are aware that these methods are based on different assumptions, but all of these are expected to estimate the "true" phylogeny in the absence of long-branch-attraction: genetic distance calculation, Maximum Parsimony (MP), and Neighbor-Joining (NJ) analysis.

Results

Our pilot phylogeny yielded a number of trees as we explored various schemes of outgroup choice and transition:transversion:gap weighting. Figs. 1-2 present the resulting phylogenetic trees under MP and NJ algorithms (tr:tv weighting 3:1) which exhibited the best statistical support for maximal number of clades. Numbers under clades represent bootstrap support based on 1000 replicates. Fig. 3 presents genetic distance matrix (uncorrected "p") for 23 studied species. Details of tree parameters are given in the figure legends. Below, we discuss the obtained topologies, their phylogenetic implications, and formulate hypotheses for further study.
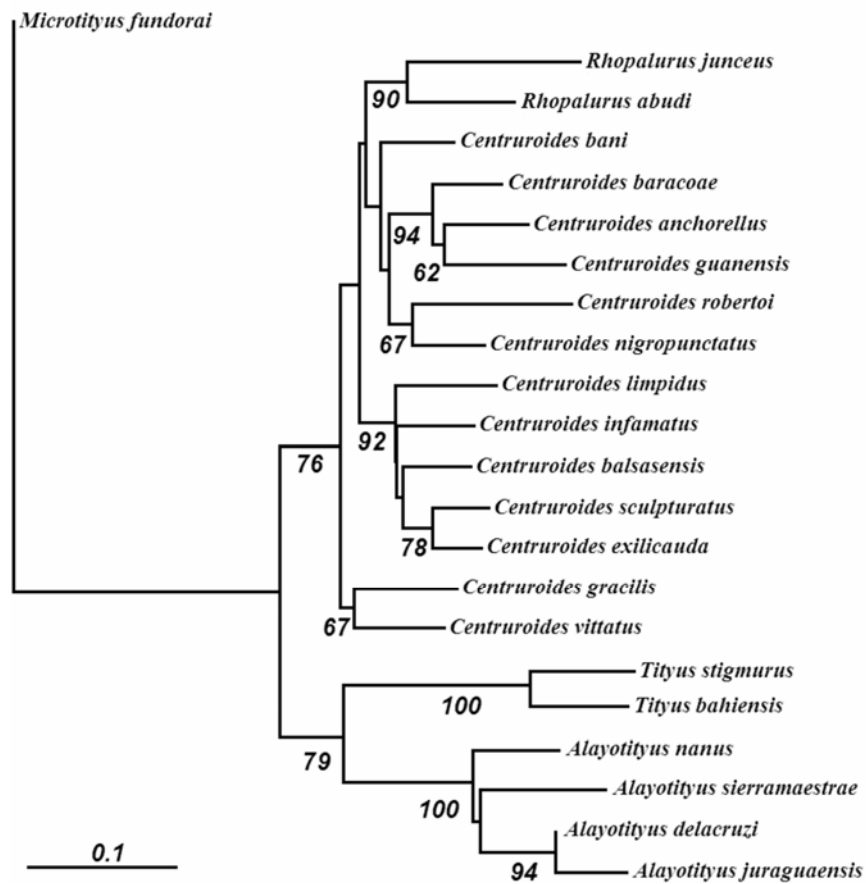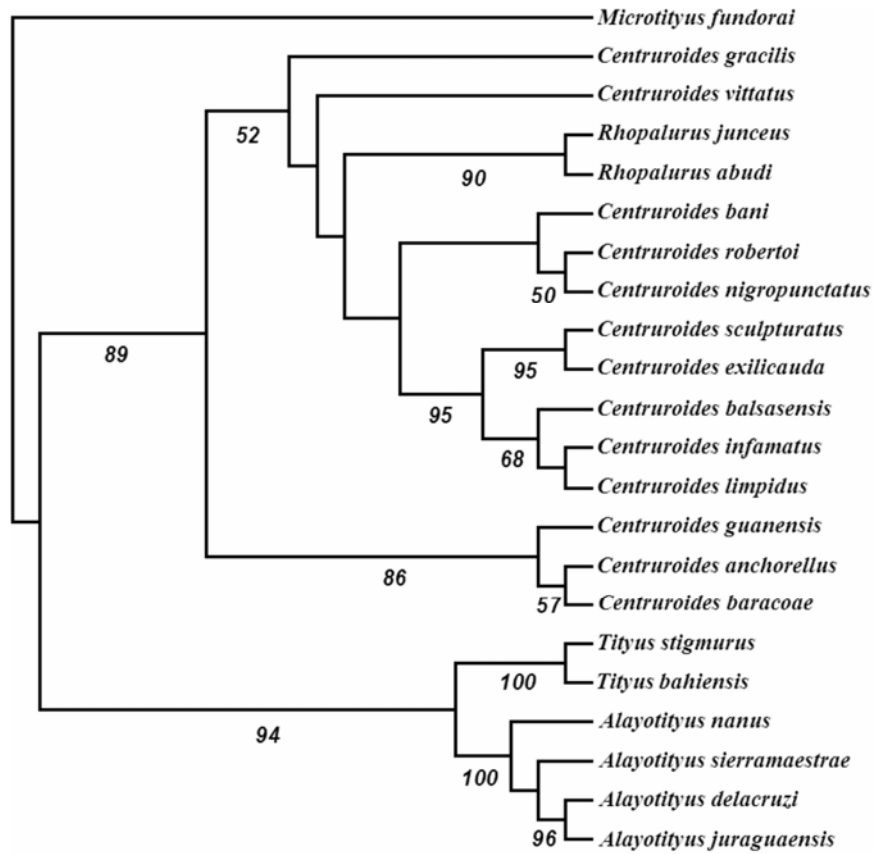
Fig. 1. MP tree; TR:TV weighting 3:1; *Microtityus* as outgroup. Of 410 total characters, 153 are constant, 181 are parsimony-informative. The single MP tree was recovered, length 738 steps. CI = 0.549; HI = 0.451. Bootstrap values > 50% (parsimony criterion) indicated under clades. **Fig. 2.** NJ tree; TR:TV weighting 3:1. *Microtityus* as outgroup. Bootstrap values >50% (distance criterion) indicated under clades.

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            1       2       3       4       5       6       7       8       9       10      11
 1 Mfun     -
 2 Rjun   0.265     -
 3 Rabu   0.261   0.163     -
 4 Cban   0.237   0.169   0.136     -
 5 Canc   0.268   0.200   0.199   0.144     -
 6 Cbar   0.256   0.190   0.184   0.120   0.093     -
 7 Cgua   0.281   0.246   0.209   0.161   0.127   0.133     -
 8 Crob   0.293   0.228   0.195   0.150   0.154   0.168   0.190     -
 9 Cnig   0.285   0.215   0.157   0.103   0.152   0.136   0.168   0.138     -
10 Cscu   0.268   0.201   0.157   0.126   0.158   0.155   0.194   0.185   0.122     -
11 Cbal   0.250   0.194   0.159   0.136   0.169   0.152   0.186   0.193   0.119   0.087     -
12 Cinf   0.250   0.192   0.166   0.129   0.153   0.149   0.183   0.180   0.134   0.101   0.095
13 Clim   0.252   0.190   0.169   0.136   0.187   0.169   0.193   0.189   0.167   0.115   0.102
14 Cexi   0.238   0.193   0.161   0.128   0.161   0.146   0.165   0.185   0.133   0.068   0.099
15 Cgra   0.216   0.191   0.171   0.136   0.177   0.151   0.176   0.231   0.158   0.151   0.152
16 Cvit   0.240   0.217   0.154   0.134   0.175   0.141   0.170   0.183   0.131   0.139   0.139
17 Tsti   0.301   0.323   0.305   0.282   0.309   0.286   0.341   0.327   0.301   0.283   0.286
18 Tbah   0.300   0.310   0.294   0.284   0.305   0.289   0.336   0.329   0.294   0.275   0.272
19 Adel   0.287   0.276   0.245   0.230   0.263   0.244   0.267   0.249   0.275   0.261   0.257
20 Ajur   0.332   0.324   0.260   0.267   0.307   0.278   0.308   0.292   0.283   0.289   0.284
21 Anan   0.286   0.276   0.260   0.246   0.270   0.258   0.303   0.259   0.280   0.266   0.257
22 Asie   0.317   0.337   0.299   0.272   0.274   0.286   0.291   0.253   0.310   0.307   0.310


            12      13      14      15      16      17      18      19      20      21      22
12 Cinf     -
13 Clim   0.110     -
14 Cexi   0.104   0.123     -
15 Cgra   0.142   0.153   0.135     -
16 Cvit   0.138   0.157   0.127   0.115     -
17 Tsti   0.278   0.305   0.282   0.282   0.282     -
18 Tbah   0.290   0.291   0.281   0.284   0.271   0.122     -
19 Adel   0.251   0.267   0.238   0.241   0.227   0.274   0.266     -
20 Ajur   0.282   0.305   0.280   0.276   0.267   0.270   0.281   0.040     -
21 Anan   0.236   0.248   0.238   0.258   0.247   0.274   0.273   0.121   0.121     -
22 Asie   0.286   0.307   0.280   0.285   0.276   0.324   0.316   0.145   0.164   0.143     -
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Fig. 3. Distance matrix (uncorrected "p")

Discussion

The New World Buthidae are considered monophyletic (Fet *et al.*, 2005) but it is unclear which group of Old World Buthidae is the most closely related to the New World lineage. The initial Old World outgroup for our phylogenetic analysis was a Madagascan endemic, *Grosphus madagascariensis* (Gervais, 1843). The preliminary phylogeny of Buthidae (Fet *et al.*, 2003) based on the 16S rRNA gene showed that two genera of New World Buthidae (*Centruroides* and *Rhopalurus*) grouped with the Madagascan *Grosphus* and Southeast Asian *Lychas*, as opposed to another well-supported clade of Old World scorpions. We explored the effect of inclusion/exclusion of different outgroups on the ingroup topology. When *Grosphus* was used as an outgroup, *Microtityus* (*Parvabsonus*) *fundorai* grouped separately from all other 21 New World sequences. Two further clades were well-supported: (*Tityus + Alayotityus*) and (*Centruroides + Rhopalurus*). When *Grosphus* was excluded, and the tree was rooted with *Microtityus*, support for these two clades and many subclades considerably increased. Therefore we proceeded with further analysis of New World genera only (22 sequences), using *Microtityus* as an outgroup. Below, we discuss major observations on the obtained phylogenies, which include various issues pertinent to phylogenetic position of 12 Cuban species and four genera to which they belong.

1. Monophyly of the clades (*Rhopalurus + Centruroides*)

and (*Alayotityus + Tityus*) was *always* supported with very high bootstrap values (89-94% in MP, 76-79% in NJ), while *Microtityus fundorai* always formed a separate, third clade. *Alayotityus* and *Microtityus* are clearly two separate, ancient lineages in the Caribbean scorpiofauna (*Alayotityus* is endemic to Cuba while *Microtityus* is found both in South America and the Caribbean). It should be noted that *Microtityus* is currently divided in two subgenera: *Microtityus* s.str. (four South American species), and *Parvabsonus* Armas, 1974 (12 Caribbean species), but only the latter was available for the present study.

The origin and phylogenetic relatives of both *Alayotityus* and *Microtityus* still remain obscure, as they have a morphology which neither matches each other nor any of the other New World genera except *Tityopsis* Armas 1974 (which seems to be very closely related to *Alayotityus*) and *Mesotityus* (which, based exclusively on literature data, could be related to *Microtityus*). In our preliminary analysis, the genus *Alayotityus* (but not *Microtityus*) clearly formed a sister group to two Brazilian species of *Tityus*. However, the phylogenetic relationships within the highly diverse, mostly South American genus *Tityus* (currently with five subgenera and 175 species: Lourenço, 2006) are unknown, and thus its phylogenetic relationships with other South American and Caribbean genera are not defined.

On the other hand, *Centruroides* and *Rhopalurus* are very closely related (i.e., they are the only known buthid

genera, which share derived supernumerary granules on the pedipalp fingers). In fact, they were considered synonyms in the past (Kraepelin, 1891; Meise, 1933), but at the same time Hoffmann (1938) considered them as widely different in origin, evolutionary history, and dispersal routes. In Hoffmann's hypothesis, *Centruroides* originated in North America, experienced an "explosive" diversification (it currently includes about 60 confirmed species and subspecies) and expanded its geographical range southwards and eastwards to Antilles and South America. *Rhopalurus* was believed to have originated in South America, where it is much less diverse (about 10 valid species) and dispersed only northward to reach the Greater Antilles. Monophyly of *Centruroides*, however, is not supported (see below), thus *Rhopalurus* could be closer to some species groups of *Centruroides* than to others.

Two species of *Rhopalurus* from the Caribbean (Cuban *R. junceus* and Hispaniolan *R. abudi*) formed a well-supported (90% in both MP and NJ) clade in our analysis, which is consistent with low diversity of this genus proposed by Hoffmann (1938). After the most recent works conducted on this genus (Armas, 1999; Teruel, 2006a; Teruel & Armas, 2006), *Rhopalurus* currently includes 19 species, almost evenly divided between two disjunct geographical areas: South America (Colombia, Venezuela, the Guyanas and Brazil: 10 species, including the type species of the genus) and the Greater Antilles (Cuba and Hispaniola: nine species). On the basis of external morphology alone, neither of these two areas seem to contain a closely related group of taxa, and at least two lineages can be roughly defined in each one (Teruel, 2006a; pers. obs.). Consequently, further studies on this topic are fully warranted.

2. Maybe the most striking finding of this study is that monophyly of *Centruroides* (13 species studied) was *never* supported. The (*Rhopalurus* + *Centruroides*) clade contained one well-supported subclade with two species of *Rhopalurus*, from two to four supported *Centruroides* subclades, and some polytomic subclades of *Centruroides*. Such results are not unexpected, since diagnostic characters that could support monophyly of *Centruroides* were never formulated. *Centruroides* is a large and morphologically complex genus, most likely a paraphyletic group, and a good "candidate for splitting". Such procedure is not new, and has already been attempted at the level of species-groups for some geographical areas such as Mexico (Hoffmann, 1932) and Cuba (Teruel, 2001b). Nevertheless, morphological characters used by these authors to separate their groups of species are not as discernable as one would wish to support upgrading these groups to genus level, and, at least in the case of the Mexican species, they have yet to be revised and updated. In the case of Cuban *Centruroides*, the morphological distinction among its three autochthonous species-groups has been hypothesized to reflect not only the existence of three separate lineages, but also the occurrence of different, possibly non-synchronous colonization events.

3. The "Mexican clade" containing five species of *Centruroides* (*C. balsasensis*, *C. exilicauda*, *C. infamatus*, *C. limpidus*, and *C. sculpturatus*) has been extremely well supported in all analyses (92-95% in both NJ and MP). In addition, *C. exilicauda* and *C. sculpturatus* (until recently considered synonyms; but see Valdez-Cruz *et al.*, 2004) always formed a very well supported subclade within the five Mexican species clade. *C. sculpturatus* is the only species of this clade which also inhabits the USA. For more detailed intraspecific phylogenies for these species, see Gantenbein *et al.* (2001) and Towler *et al.* (2001).

This result is fairly consistent with morphology, as these five Mexican species represent a very homogeneous group of *Centruroides* with the same basic color pattern and pectinal tooth count, similar carination and granulation, and comparable pedipalp and metasoma morphology. This group as a whole shows a compact distribution all over Mexico and southwestern USA. Further DNA studies on other Mexican and Central American species (*C. baergi*, *C. elegans*, *C. hoffmanni*, *C. meisei*, *C. koesteri*, *C. orizaba*, *C. pallidiceps*, and even *C. nigrovariatus*, *C. noxius*, and the problematic subspecies *C. elegans insularis*, *C. infamatus ornatus*, and *C. limpidus tecomanus*) are urgently needed: these taxa show most of (or sometimes all) the abovementioned morphological and distributional features (R. Teruel, pers. obs.; see also Hoffmann, 1932, 1938; Armas & Martín, 1999, 2003; Armas *et al.*, 1995, 2003; Martín *et al.*, 2005), suggesting that they could well belong in the "Mexican clade" discussed herein.

4. No single "Caribbean clade" for *Centruroides* has been recovered. In some analyses six Caribbean species (excluding *C. gracilis*) formed a clade (Fig. 2) but it had no statistical support.

However, a well-supported clade (86% in MP, 94% in NJ) was recovered for three Caribbean species of *Centruroides*: *C. guanensis*, *C. baracoae*, and *C. anchorellus*. The first species is a Caribbean subendemic (it occurs in Cuba and the Bahamas, but it is also found in extreme southern Florida, including the Florida Keys). The latter two are endemic to Cuba and were once considered synonyms (Armas, 1984), but recently Teruel (2000) demonstrated both to be separate species based on morphological grounds. The present study confirms that *C. anchorellus* and *C. baracoae* are different but closely related species; the genetic distance between both is 9.3%, a value lying within the range herein found for other pairs of *Centruroides* such as *C. exilicauda*/*C. sculpturatus* (6.8%), *C. gracilis*/*C. vittatus* (11.5%), and *C. robertoi*/*C. nigropunctatus* (14%) (Fig. 3). Relationships within this clade are less defined: while MP had moderate (57%) support for (*C. baracoae* + *C. anchorellus*) clade, NJ had also a moderate (62%) support for (*C. guanensis* + *C. anchorellus*) clade. It would be very interesting to conduct a similar study on the Cuban endemic *C. stockwelli*, the other species included in the "*anchorellus*" species group by Teruel (2000). Furthermore, the Hispaniolan endemic *Centruroides marcanoi* possibly belongs in this clade as it strongly resembles *C. guanensis* in morphology (R. Teruel, pers. obs.), so further DNA studies on this species are warranted.

An additional Cuban clade (67% support in NJ) was recovered for *C. robertoi* and the newly described *C. nigropunctatus* (R. Teruel, this volume); this clade grouped outside of the clade containing three other species. A separate position of the (*C. robertoi* + *C. nigropunctatus*) clade with respect to the (*C. guanensis* + *C. anchorellus* + *C. bara-*

coae) clade is consistent with the morphology-based study of Teruel (2001b) on Cuban *Centruroides*, where *C. robertoi* was separated in its own monotypic group (*C. nigropunctatus* was still undescribed at this time) and considered a very ancient Antillean element of the genus, maybe the oldest. The "*robertoi*" species group was originally established as monotypic, but the new addition of *C. nigropunctatus* showed it to be more diverse than previously suspected. Also, the southern Hispaniolan and Navassan endemics, *Centruroides alayoni* and *C. luceorum,* were explicitly associated to *C. robertoi* in their original descriptions (Armas, 1999). Such an assumption, however, still needs to be verified by more detailed studies (R. Teruel, pers. obs.). Genetic distance of ca. 14% (Fig. 3) between *C. robertoi* and *C. nigropunctatus* confirms that these are separate, although closely related, endemic Cuban species.

The species *C. bani* (Dominican Republic) did not group closely with any other Caribbean species, and requires further investigation with the addition of its closest Hispaniolan relatives (i.e., *C. nitidus* and the enigmatic *C. tenuis*). From morphology alone, it is not yet possible to discern a possible match for these three species among the "continental" *Centruroides*, even though they show some apparent resemblance to the "Mexican clade" herein discussed.

No DNA information on *Centruroides* from any other Caribbean islands exists, and is crucial to further understanding of this diverse genus, as there are at least 25 valid species and subspecies of *Centruroides* which are endemic to this geographical area.

5. The North American species *Centruroides vittatus* and Cuban *C. gracilis* grouped outside of the Mexican clade and Caribbean species; in NJ analysis they formed a supported clade (67%). *C. gracilis*, therefore, is not closely related to any of the other six studied Caribbean species of *Centruroides*. It could be a separate Cuban autochthonous lineage; or, alternatively, it could be *introduced* to Cuba; its current geographical distribution inside Cuba supports *C. gracilis* as an introduced scorpion (it is almost strictly synanthropic, occurring mainly inside and/or near human settlements). These hypotheses should be tested by comparing DNA sequences of Cuban and Mexican/Central American specimens of *C. gracilis*.

6. Among *Alayotityus* species, a very high support (94-96%) is seen for the clade (*A. juraguaensis* + *A. delacruzi*). These two species are extremely close to each other (only 4.0% genetic distance), when compared to the distances between other congeneric species (12.1-16.4%). The relationship between this clade and the other two species (*A. sierramaestrae*, *A. nanus*) is not clear.

The topologies obtained from the various trees for the internal structure of *Alayotityus* exactly match the previous morphology-based analysis conducted by Teruel (2001a, 2002, 2004). The close distance between *A. juraguaensis* and *A. delacruzi* is consistent with their hypothesized origin (Teruel, 2001a), with the trogloxene *A. juraguaensis* and the non-troglomorphic troglobite *A. delacruzi* being derived by isolation of the latter inside Cueva de los Majáes cave (Vachon, 1977). According to Nicasio Viña Bayés (pers. comm.), consecutive Pleistocene interglacial elevations of

sea level inundated the cave. Since the last of these elevations occurred ≈ 80,000 years ago, this timeline obviously sets the maximum possible age for the separation of these two species. On morphological grounds, it is not easy to define whether one species derives directly from the other or both have diverged from a common ancestor. Nevertheless, there is some evidence which suggests that *A. delacruzi* is indeed nearest to the presumed ancestor: (a) it shows a single autapomorphy among the members of the genus (pedipalps with all carinae outlined in deep red), while *A. juraguaensis* shows at least three (elongated metasoma and pedipalps, reduction of overall granulation and highest pectinal tooth count); (b) species of *Alayotityus* are mainly forest-dwelling scorpions which live in humid refuges (i.e., leaf litter), so the present distribution of *A. delacruzi* (restricted to a wet cave that forms an enclave inside a semidesert coastal area) seems to be of relict character. Even though the genetic distance between *A. delacruzi* and *A. juraguaensis* is somewhat low (4%) when compared to that obtained herein for other species of the genus (12.1-16.4%), we consider that it warrants full species status for the former because it is also supported by both morphology (both species fit into one of the most easy-to-separate diagnostic key couplets in the genus) and ecology (they are strictly parapatric: *A. delacruzi* lives only inside this cave, which is located on the highest level of the coastal limestone terraces, but *A. juraguaensis* occupies the xeric lower levels; for a general characterization of the entire area, see Fong *et al.*, 2005).

On the other hand, the fact that the (*A. nanus* + *A. sierramaestrae*) clade was not supported is in full agreement with Teruel (2002, 2004), who erected the "*Alayotityus nanus*" species complex to accommodate *A. nanus* and three other species which lack trichobothrium *esb* on the fixed finger of pedipalp, as opposed to the remaining species (including *A. sierramaestrae*), which possess this trichobothrium. Neobothriotaxy in Buthidae is very rare (Vachon, 1977; Soleglad & Fet, 2001), and the derived loss of *esb* is most likely a unique synapomorphy for this species complex. Further, detailed DNA-based investigation of this endemic Cuban genus, which currently includes eight described species, is warranted.

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