



Phylogenomics facilitates stable scorpion systematics: Reassessing the relationships of Vaejovidae and a new higher-level classification of Scorpiones (Arachnida)

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ABSTRACT

The Neartic family Vaejovidae (Scorpiones: Chactioidea) has long been treated as a diverse and systematically cohesive group of scorpions, but its monophyly and relationship to other scorpion families have historically been questioned. Morphological data have supported its monophyly and a variety of phylogenetic placements within the superfamily Chactioidea. Recent phylogenomic analyses have instead recovered vaejovids as polyphyletic (albeit with minimal taxonomic sampling) and Chactioidea as paraphyletic. Here, we reexamined the monophyly and phylogenetic placement of the family Vaejovidae, sampling 17 new vaejovid libraries using high throughput transcriptomic sequencing. Our phylogenomic analyses revealed a previous misplacement of *Smeringurus me-saensis*. Regardless, we recovered Vaejovidae as diphyetic due to the placement of the enigmatic genus *Uroctonus*. The remaining vaejovids formed a clade that was strongly supported as the sister group of the superfamily Scorpionoidea, a placement insensitive to matrix completeness or concatenation vs. species tree approaches to inferring the tree topology. Chactioidea was invariably recovered as a paraphyletic group due to the nested placement of Scorpionoidea. As first steps to resolving the paraphyly of Chactioidea, we take the following systematic actions: (1) we establish the superfamily Superstitionioidea (**new superfamily**) to accommodate Superstitioniidae; (2) we restore Vaejovoidea (**status revalidated**) as a valid superfamily that excludes *Uroctonus*; and (3) we treat the families Caraboctonidae, Troglotayosicidae, and the subfamily Uroctoninae as *incertae sedis* with respect to superfamilial placement. Our systematic actions thus establish the monophyly of the presently redefined Chactioidea and Vaejovoidea.

1. Introduction

In recent years, the higher-level systematics of scorpions has been significantly revised. Phylogenomic analyses established the two basal branches of scorpions, the parvorders Buthida and Iurida (Sharma et al., 2015, 2018). While the composition of Buthida – as well as the monophyly of its non-monotypic families – were strongly supported, numerous lineages within Iurida were shown to be non-monophyletic and exhibit unanticipated phylogenetic placement. These included the traditional Iuroidea, due to the diphyly of *Iurus* and *Hadrurus*,

Scorpionoidea, due to the diphyly of bothriurids and the remaining scorpionoids, and the superfamily Chactioidea, due to the nested placement of Scorpionoidea. Most curious among the chactoid families in that study was the phylogenetic resolution of the Vaejovidae. Scorpions of this family, which harbors nearly 220 species, are distributed in North America, with its peak of diversity found in Mexico (González-Santillán and Prendini, 2013, 2015a, 2018; Santibáñez-López et al., 2015).

The systematics of Vaejovidae is in a state of flux. On the basis of several morphological analyses (e.g. Soleglad and Fet, 2003, 2006,

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Table 1

Generic and suprageneric classification of the North American scorpion family Vaejovidae Thorell, 1876 proposed by Stockwell (1989), modified by Soleglad and Fet (2008) and González-Santillán and Prendini (2013, 2015b).

Syntropinae Kraepelin, 1905
<i>Balsateres</i> González-Santillán and Prendini, 2013
<i>Chihuahuanus</i> González-Santillán and Prendini, 2013
<i>Kochius</i> Soleglad and Fet, 2008
<i>Kuarapu</i> Francke and Ponce-Saavedra, 2010
<i>Maaykuyak</i> González-Santillán and Prendini, 2013
<i>Mesomexovis</i> González-Santillán and Prendini, 2013
<i>Paravaejovis</i> Williams, 1980
<i>Syntropis</i> Kraepelin, 1900
<i>Thorellius</i> Soleglad and Fet, 2008
<i>Vizcaino</i> González-Santillán and Prendini, 2013
Smeringurinae Soleglad and Fet, 2008
<i>Paruroctonus</i> Werner, 1934
<i>Smeringurus</i> Haradon, 1983
<i>Vejovoidus</i> Stahnke, 1974
Vaejovinae Thorell, 1876
<i>Franckus</i> Soleglad and Fet, 2005
<i>Pseudouroctonus</i> Stahnke, 1974
<i>Uroctonus</i> Thorell, 1876
<i>Uroctonites</i> Williams and Savary, 1991
<i>Vaejovis</i> C.L. Koch, 1836

2008; Prendini and Wheeler, 2005; Fet et al., 2006), Vaejovidae is held to comprise three subfamilies, Smeringurinae, Syntropinae, and Vaejovinae (Table 1), as well as some genera regarded as *incertae sedis* (e.g., *Gertschius*, *Serradigitus*, *Stahnkeus*, and *Wernerius*). Of the three established subfamilies, only the monophyly of Syntropinae (the most

diverse division of vaejovids with 11 genera and nearly 60 species), has been recently tested using morphological and molecular data (González-Santillán and Prendini, 2013; González-Santillán and Prendini, 2015b). In addition, the phylogenetic position of the North American genus *Uroctonus* remains in dispute. Previous analyses (e.g. Stockwell, 1989; Sissom, 1990; Prendini and Wheeler, 2005) recovered this genus as a basally branching vaejovid. Others (Soleglad and Fet, 2003, 2004) included *Uroctonus* in its own subfamily within the largely Neotropical family Chactidae. Higher level systematics based on cladistic analyses of morphological character systems have recovered Vaejovidae as the sister group to Chactidae (Lamoral, 1980), Iuridae (Stockwell, 1989; Sissom, 1990; Coddington et al., 2004), or as the sister group to the remaining Chactoidea (Soleglad and Fet, 2003) (Fig. 1). Generally, however, morphological cladistic analyses have never questioned the monophyly of Vaejovidae.

It was therefore odd that the first phylogenomic analysis of scorpions (i.e. Sharma et al., 2015) and a subsequent analysis with greater sampling of basal Iurida (Sharma et al., 2018), recovered Vaejovidae as diphylectic, due to the position of *Smeringurus mesaensis* (Vaejovidae: Smeringurinae) as sister taxon to *Superstitionia donensis* (Superstitioniidae). The only other vaejovid in that analysis, *Paravaejovis spinigerus* (Syntropinae), was recovered as the sister group to Scorpionoidea (*sensu* Sharma et al., 2015) (Fig. 1). However, reexamination of the *Smeringurus mesaensis* library suggested that this dataset may have been contaminated due to the inclusion of gut tissue, and it was removed from later analyses (Santibáñez-López et al., 2018). As the *Paravaejovis spinigerus* transcriptome was the only remaining exemplar of Vaejovidae in that analysis, the monophyly of this family was untested in a phylogenomic context, and the hypothesis of its placement as

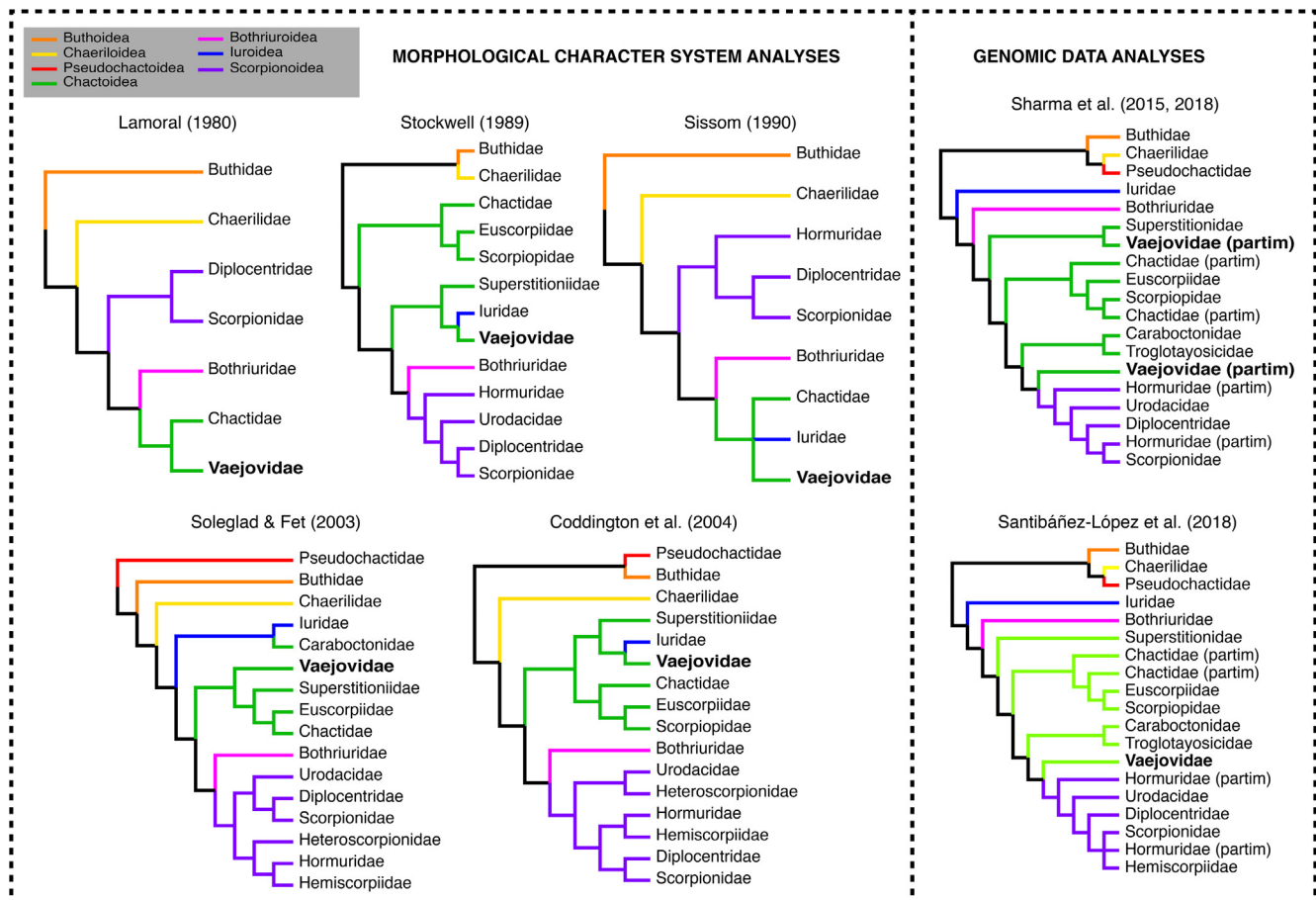


Fig. 1. Historical hypotheses of scorpion relationships. Colors in tree topologies correspond to superfamilies (top left). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sister group to Scorpionoidea also remains questionable because of the lack of a thorough empirical assessment. This uncertainty hinders hypothesis testing in studies of morphological evolution (e.g. Monod et al., 2017) and venom diversification (e.g. Santibáñez-López et al., 2018), and may also affect selection of appropriate outgroups for downstream analyses, including divergence time estimation for biogeographic inference (Bryson et al., 2013).

In order to assess the phylogenetic validity of Vaejovidae and Chactioidea more generally, we sequenced the transcriptomes of 17 vaejovid species, including (1) a new library of *S. mesaensis*, (2) a new library of its congener *Smeringurus vachoni*, and (3) two libraries of *Uroctonus mordax*. We also included a published library of *Superstitionia donensis* generated in a separate laboratory as an internal control for the possibility of contamination of *Superstitionia donensis* as well in the study of Sharma et al. (2015). These libraries were added to the existing phylogenomic dataset of all major scorpion families (Sharma et al., 2015, 2018). We inferred gene orthology *de novo* and inferred relationships using matrices of varying size and completeness. Here we show that all non-uroctonine Vaejovidae are monophyletic and sister group to Scorpionoidea. We undertake a series of taxonomic actions to begin redressing the paraphyly of Chactioidea.

2. Materials and methods

2.1. Taxon sampling

Living specimens were collected by hand during the day under rocks or other objects on the ground, or with the aid of ultraviolet lamps at night from several localities in Mexico and USA, or from captive bred colonies (Tables 2 and 3). Scorpions collected from the field trips were dissected into RNAlater solution (Ambion); living specimens were anesthetized using CO₂ and vivisected in 1 × PBS (phosphate buffered saline). From all specimens, the brain, legs, and telson were dissected for sequencing; midgut and hindgut tissues were avoided to reduce the possibility of gut content sequencing and contamination. Total RNA was extracted and sequenced using previously described protocols (e.g. Sharma et al., 2015). Transcriptomes previously published by us were included for outgroups sampling (Sharma et al., 2014, 2015, 2018; Table 3).

2.2. Orthology inference and phylogenomic reconstruction

The search for orthologous sequences to infer species tree was conducted *de novo* using the phylogenetically informed orthology criterion implemented in UPhO (Ballesteros and Hormiga, 2016). All transcriptomes were combined and served as database and query at the same time (“all vs all” strategy) using *blastp*. This strategy was favored over the “query search” to improve the changes to obtain more orthologous sequences. Sequences were clustered in gene families using *mcl* (Dongen, 2000; Enright et al., 2002). The full variety of values for the inflation parameter ($i = 1.4, 2, 4, 6$) were explored, with the selection of the clustering produced with $i = 6$ based on the efficiency scores reported by *mcl*. A total of 8833 clusters produced with at least 30 species (64% of the dataset) was carried for downstream analyses.

Gene-family trees (GFTs) were estimated for each cluster using IQtree v 1.5.5 (Nguyen et al., 2014) following multiple sequence alignment with MAFFT v 7.0 (Katoh and Standley, 2013), gap masking with trimAl v1.2 (Capella-Gutiérrez et al., 2009), and removing sequences with less than 50 amino acids or less than 25% unambiguous sites after trimming (using the script *Al2Phylo.py* from UPhO). All analyses were parallelized implemented through *gnu-parallel* (Tange, 2011). Loci were filtered using three thresholds for taxon occupancy: 64% (loci with sequence data for at least 30 species; henceforth Matrix 1); 74% (loci with sequence data for at least 35 species; henceforth Matrix 2), and 89% (loci with sequence data for at least 42 species; henceforth Matrix 3), with 75% of nodal support and retaining in-

paralogs ($-m \{30,35,42\} -S 0.75 -iP$), using UPhO (Ballesteros and Hormiga, 2016). The individual orthogroups were aligned and trimmed as mentioned above. They were then concatenated in supermatrices with the script *geneStitcher.py* (Ballesteros and Hormiga, 2016). In-paralogs, alleles, duplicates and/or splice-variants retained in the orthogroups were resolved in favor of the longest sequence.

Preliminary analysis was conducted using all three *Smeringurus* libraries (*Smeringurus mesaensis* from Sharma et al., 2015 and this study; *Smeringurus vachoni* from this study). As this result showed a dubious placement of the *S. mesaensis* library from 2015 (Results, below), it was removed from all subsequent analyses and the new *S. mesaensis* library was retained. The final dataset therefore consisted of 45 scorpion species and two non-scorpion arachnid outgroups (one spider, one horse-shoe crab).

All matrices were partitioned by locus, selecting the best-fitting amino acid-substitution model per partition with four gamma categories detected with ModelFinder (Kalyaanamoorthy et al., 2017). Maximum likelihood (ML) analyses of the three matrices were performed using IQtree implementing ultrafast bootstrap resampling (Minh et al., 2013). Species tree estimation of the constituent orthogroups of the three matrices were generated using the ML gene tree topologies and ASTRAL-II (Mirarab and Warnow, 2015).

3. Results

3.1. Phylogenetic placement of re-sequenced *Smeringurus* and *Superstitionia* terminals

Orthology inference using UPhO recovered the following numbers of loci for each matrix: 2742 genes, 1,271,890 amino acid sites and 23% missing data (Matrix 1); 1730 genes, 753,997 amino acid sites and 16% missing data (Matrix 2); and 607 genes, 243,560 amino acid sites and 6% missing data (Matrix 3). These values reflect the composition of the matrices after exclusion of an older dubious library of *Smeringurus mesaensis* (from Sharma et al., 2015), which was recovered as a basally branching vaejovid in a preliminary species tree analysis or as sister taxa to Superstitioniidae (Supplementary Figs. 1–2), rather than clustering with its congeners and its conspecific. This phylogenetic placement, together with its recovery as sister group to *Superstitionia donensis* in supermatrix analyses (Sharma et al., 2015, 2018) is highly suggestive of contamination of the 2015 *Smeringurus mesaensis* library by *Superstitionia donensis*, possibly due to gut tissue contamination in the former, as the two species are sympatric. This library was excluded from all analyses thereafter. By contrast, both terminals of *Superstitionia donensis* clustered together with nearly-zero branch lengths, consistent with on-target identification and sequencing of conspecific terminals.

3.2. Higher level relationships

ML analyses and species tree reconciliation of Matrices 1–3 invariably recovered the higher level relationships previously obtained by phylogenomic analyses (Sharma et al., 2015, 2018; Santibáñez-López et al., 2018) with respect to (1) mutual monophyly of Buthida and Iurida; (2) the distant relationship of Scorpionoidea and its erstwhile constituent Bothriuroidea (*sensu* Sharma et al., 2018); (3) the non-monophyly of Chactioidea; and (4) a basal grade of Iurida comprised of Iuridae, Bothriuridae, and Supersitioniidae (Figs. 2 and 3). Other relationships corroborated by this dataset include the distant relationship of *Iurus* and *Hadrurus* – previously held to be part of the superfamily Iuroidea – and the clustering of *Hadrurus* with the troglobitic *Belisarius xambeui* (Troglotayosicidae) in addition to *Uroctonus mordax* (Fig. 3; see below).

3.3. Phylogenetics of Vaejovidae

With respect to ingroup relationships, all ML analyses uniformly

Table 2
Collecting localities of newly sequenced scorpions.

Species	Locality/Region of origin	Latitude	Longitude	Date	Collector
<i>Chihuahuana coduillae</i>	Mexico: Zacatecas: Municipio Concepción del Oro, Anahuac.	24.531	–101.3192	iv.2015	H. Carmona
<i>Konetontli acapulco</i>	Mexico: Guerrero: Municipio Acapulco de Juárez, Cumbres de Llano Largo.	16.8285	–99.8489	29.i.2015	J.G. Baldazo, R. Teruel, M.Flores, J. Cortéz, E. Zarate, A. Balanzar
<i>Konetontli chamelaensis</i>	Mexico: Jaisco: Municipio La Huerta, Estación Biológica Chamela.	19.504	–105.0379	30.ii.2015	J. López, E. González-Santillán
<i>Kovarkia bogerti</i>	USA: California: Riverside County: San Jacinto Mountains, Idyllwild-Pine Cove, pine forest with granitic boulders.	n/a	n/a	14.vii.2016	E. González-Santillán, L. Monod, W. Savary
<i>Kuarapu purhepecha</i>	Mexico: Michoacán: Municipio La Huacana, marker Km 17 road between Zicuacán and Churumuco (type locality).	18.8142	–101.9159	16.v.2015	E. Oliveros, J. Ponce, A. Quijano, J. Maldonado, E. González-Santillán
<i>Mesomexovis occidentalis</i>	Mexico: Guerrero, Municipio Acapulco de Juárez, Cumbres de Llano Largo.	16.8285	–99.8489	29.i.2015	J.G. Baldazo, R. Teruel, M.Flores, J. Cortéz, E. Zarate, A. Balanzar
<i>Mesomexovis</i> aff. <i>variegatus</i>	Mexico: Michoacán: Municipio La Huacana, marker Km 17 road between Zicuacán and Churumuco.	18.8142	–101.9159	16.v.2015	E. Oliveros, J. Ponce, A. Quijano, J. Maldonado, E. González-Santillán
<i>Paruroctonus baergi</i>	South Carolina, USA.	n/a	n/a	iii.2018	Captive bred
<i>Plesiochactas dilutus</i>	Mexico: Oaxaca, Santa María Tlahuitoltepec, under logs.	n/a	n/a	28-vii-2017	C.E. Santibáñez-López, N. Gómez
<i>Pseudouroctonus apacheanus</i>	USA: Arizona: Cochise County: Chiricahua Mountains, Sunny Flat trail, 1502 m, canyon with large granitic boulders, forest with predominant oak, along dry creek.	n/a	n/a	11.vii.2016	E. González-Santillán, L. Monod.
<i>Scorpio fuscus</i>	Israel: Banias, field school's fence adjacent to Cabin 73; blacklighting.	33.2361	35.6722	11-vi-2017	P.P. Sharma, S. Aharon, I. Armiach, E. Gavish-Regev
<i>Smeringurus mesasiensis</i>	South Carolina, USA.	n/a	n/a	iii.2018	Captive bred
<i>Smeringurus vachoni</i>	South Carolina, USA.	n/a	n/a	iii.2018	Captive bred
<i>Thorellius intrepidus</i>	Mexico: Colima: Municipio Coquimatlan, El Palapo.	19.1985	–103.9138	3.iv.2015	J. Valencia, J. López, E. González-Santillán
<i>Uroctonites huachuca</i>	USA: Arizona: Cochise County: Coronado National Forest, Huachuca Mountains, Carr Canyon road, 2057 m, on rock rock walls.	n/a	n/a	13.vii.2016	E. González-Santillán, L. Monod, W. Savary
<i>Uroctonus mordax</i> (1)	South Carolina, USA.	n/a	n/a	iii.2018	Captive bred
<i>Uroctonus mordax</i> (2)	USA: California: Napa County: S of Calistoga, in burrows, under leaf litter at the foot of trees	n/a	n/a	16.vii.2016	E. González-Santillán, L. Monod, W. Savary
<i>Vaejovis cashi</i>	USA: Arizona: Cochise County: Chiricahua Mountain, Sunny Flat Trail, 1526 m, 11.VII.2016, canyon with large granitic boulders, forest with predominant oak, along dry creek	n/a	n/a	11.vii.2016	E. González-Santillán, L. Monod.
<i>Vaejovis mexicanus</i>	Mexico: Ciudad de México: Delagación Álvaro Obregón, Pueblo de San Bartolo Ameyalco.	19.333	–99.3238	i-2015	E. González-Santillán

Table 3
List of the 45-scorpion species, plus two outgroups used in the phylogenomic analyses.

	Species	Number of reads	Contigs	SRA BioProject	SRA BioSample	SRA Accession	Method
BOTHRIUROIDEA							
Bothriuridae	<i>Bothriurus coriaceus</i>	23,289,767	196,303	PRJNA429236	SAMN08331723	SRR6467511	HiSeq 2 × 150
	<i>Centromachetes</i> sp.	5,573,242	87,052	PRJNA429234	SAMN08331856	SRR6467879	HiSeq 2 × 150
BUTHOIDEA							
Buthidae	<i>Hottentotta trilineatus</i>	9,498,355	118,058	PRJNA270678	SAMN03268330	SRR1721800	HiSeq 2 × 150
	<i>Androctonus australis</i>	15,028,017	158,114	PRJNA270676	SAMN03268328	SRR1724216	HiSeq 2 × 150
CHACTOIDEA							
Caraboctonidae	<i>Hadrurus arizonensis</i>	21,465,320	159,818	PRJNA270669	SAMN03268090	SRR1721733	HiSeq 2 × 150
	<i>Hadrurus spadix</i>	24,243,211	5568	PRJNA340270	SAMN05711364	SRR4069278	HiSeq 2 × 150
Chactidae	<i>Anuroctonus phaiodactylus</i>	21,530,977	158,446	PRJNA270683	SAMN03268340	SRR1721879	HiSeq 2 × 150
	<i>Brotheas granulatus</i>	20,318,273	134,137	PRJNA270684	SAMN03268341	SRR1721887	HiSeq 2 × 150
Euscorpidae	<i>Euscorpis italicus</i>	12,667,128	108,927	PRJNA270686	SAMN03268342	SRR1721892	HiSeq 2 × 150
	<i>Megacormus</i> sp.	37,103,196	62,656	PRJNA270687	SAMN03268343	SRR1767669	HiSeq 2 × 150
	<i>Megacormus gertschi</i>	39,638,145	110,528	PRJNA320105	SAMN04916686	SRR3657526	Illumina IIX 1 × 72
	<i>Plesiochactas dilutus</i>	42,736,309	94,208	PRJNA474017	SAMN09289874	SRR7250103	HiSeq 2 × 225
Scorpiopidae	<i>Scorpiops</i> sp.	19,929,085	272,742	PRJNA270692	SAMN03268347	SRR1767662	HiSeq 2 × 150
Superstitioniidae	<i>Superstitionia donensis</i>	23,245,173	166,551	PRJNA270688	SAMN03268346	SRR1721951	HiSeq 2 × 150
	<i>Superstitionia donensis</i>	16,145,663	219,073	PRJNA345499	SAMN05868008	SRR4381683	Illumina IIX 1 × 72
Troglotayosicidae	<i>Belisarius xambeui</i>	10,567,924	101,260	PRJNA270693	SAMN03268349	SRR1721953	HiSeq 2 × 150
Vaejovidae	<i>Chihuahuanus coahuilae</i>	28,119,786	63,104	PRJNA474038	SAMN09290524	SRR7439185	HiSeq 1 × 100
	<i>Konetontli acapulco</i>	33,525,521	96,426	PRJNA474031	SAMN09290500	SRR7422029	HiSeq 1 × 100
	<i>Konetontli chamelaensis</i>	33,479,827	86,329	PRJNA474032	SAMN09290501	SRR7427084	HiSeq 1 × 100
	<i>Kovarikia boggetti</i>	71,187,460	108,588	PRJNA517227	SAMN10822628	SRR8518584	HiSeq 2 × 150
	<i>Kuarapu purhepecha</i>	29,931,709	61,069	PRJNA474035	SAMN09290513	SRR7439043	HiSeq 1 × 100
	<i>Mesomexovis occidentalis</i>	23,508,274	50,997	PRJNA474039	SAMN09290525	SRR7439610	HiSeq 1 × 100
	<i>Mesomexovis</i> aff. <i>variegatus</i>	34,525,919	77,864	PRJNA474040	SAMN09290526	SRR7439652	HiSeq 1 × 100
	<i>Paravaejovis spinigerus</i>	8,892,658	97,571	PRJNA270694	SAMN03268351	SRR1721954	HiSeq 2 × 150
	<i>Paruroctonus baergi</i>	30,113,444	141,875	PRJNA474019	SAMN09289876	SRR7443668	HiSeq 1 × 100
	<i>Pseudouroctonus apacheanus</i>	50,784,482	111,600	PRJNA517227	SAMN10822631	SRR8518585	HiSeq 2 × 150
	<i>Smeringurus mesaensis</i>	28,918,915	121,718	PRJNA474020	SAMN09289877	SRR7473845	HiSeq 1 × 100
	<i>Smeringurus vachoni</i>	32,783,756	147,814	PRJNA474021	SAMN09289878	SRR7474136	HiSeq 1 × 100
	<i>Thorellius intrepidus</i>	28,233,147	69,834	PRJNA474033	SAMN09290512	SRR7427141	HiSeq 1 × 100
	<i>Uroctonites huachuca</i>	67,589,326	107,340	PRJNA517227	SAMN10822630	SRR8518582	HiSeq 2 × 150
	<i>Uroctonus mordax</i> (1)	47,213,766	162,715	PRJNA474018	SAMN09289875	SRR7415024	HiSeq 2 × 225
	<i>Uroctonus mordax</i> (2)	67,589,326	111,486	PRJNA517227	SAMN10822629	SRR8518581	HiSeq 2 × 150
	<i>Vaejovis cashi</i>			PRJNA517227	SAMN10822627	SRR8518583	HiSeq 2 × 150
	<i>Vaejovis mexicanus</i>	30,537,281	73,844	PRJNA474030	SAMN09290473	SRR7421527	HiSeq 1 × 100
CHAEILOIDEA							
Chaerilidae	<i>Chaerilus celebensis</i>	20,801,179	215,165	PRJNA270679	SAMN03268336	SRR1721804	HiSeq 2 × 150
IUROIDEA							
Iuridae	<i>Iurus dekanum</i>	11,774,511	76,669	PRJNA270671	SAMN03268091	SRR1721734	HiSeq 2 × 150
PSEUDOCHACTOIDEA							
Pseudochactidae	<i>Troglokhammouanus steineri</i>	20,456,204	122,671	PRJNA270673	SAMN03268326	SRR1721739	HiSeq 2 × 150
	<i>Vietbocap lao</i>	19,987,805	146,981	PRJNA270675	SAMN03268327	SRR1721740	HiSeq 2 × 150
SCORPIONOIDEA							
Scorpionidae	<i>Pandinus imperator</i>	17,620,229	139,997	PRJNA270658	SAMN03267995	SRR1721600	HiSeq 2 × 150
	<i>Scorpio fuscus</i>	28,955,555	64,185	PRJNA474016	SAMN09289873	SRR7249741	HiSeq 2 × 225
Hormuridae	<i>Liocheles australasiae</i>	15,082,229	173,567	PRJNA270659	SAMN03268000	SRR1721664	HiSeq 2 × 150
Diplocentridae	<i>Diplocentrus diablo</i>	25,583,200	201,918	PRJNA270668	SAMN03268089	SRR1721672	HiSeq 2 × 150
Urodacidae	<i>Urodacus elongatus</i>	21,820,617	245,104	PRJNA491927	SAMN10092874	SRR7885472	HiSeq 2 × 150
OUTGROUPS							
	<i>Leucauge venusta</i>	49,301,974	189,630	PRJNA236497	SAMN02597595	SRR1145740	HiSeq 2 × 150
	<i>Limulus polyphemus</i>	65,099,444	110,362	PRJNA236515	SAMN02597592	SRR1145732	HiSeq 2 × 150

recovered the monophyly of the non-uroctonine Vaejovidae as the sister group to Scorpionoidea, with consistently maximal nodal support (Figs. 2 and 3). Our analyses also supported the monophyly of Syntropinae and Smeringurinae with maximal nodal support (Fig. 2). The monophyly of Vaejovinae was not recovered due to the placement of a clade including the former uroctonoid species (*sensu* Stockwell, 1992) as sister group to the rest of the vaejovids. Relationships between these three subfamilies were uniform in our analyses (Fig. 3).

Uroctonus mordax was never recovered as part of, or sister group to, the remaining Vaejovidae or Chactidae, a result highly supported across all analyses. Rather, this species was recovered as sister taxon to *Hadrurus* (*H. arizonensis* + *H. spadix*) in all analyses. Interestingly, the clade formed by (*U. mordax* (*H. arizonensis* + *H. spadix*)) had high nodal support under Matrix 2 and was recovered as sister group to the clade

(*Belisarius xambeui* (Scorpionoidea + Vaejovidae)), also with high nodal support (Fig. 2). By contrast, only in the maximum likelihood analysis of the compact Matrix 3, *Uroctonus*, *Hadrurus*, and *Belisarius* formed a weakly supported clade (BS = 75%).

Species tree analyses also recovered the monophyly of the non-uroctonine Vaejovidae and its phylogenetic placement as sister group to Scorpionoidea (Fig. 3). All species-tree analyses recovered also Vaejovinae as paraphyletic due to the position of the former uroctonoid species and the remaining vaejovids (Syntropinae + Smeringurinae). As in the ML analyses, *U. mordax* was recovered as sister taxon to *Hadrurus* (Matrix 1–3; Fig. 3).

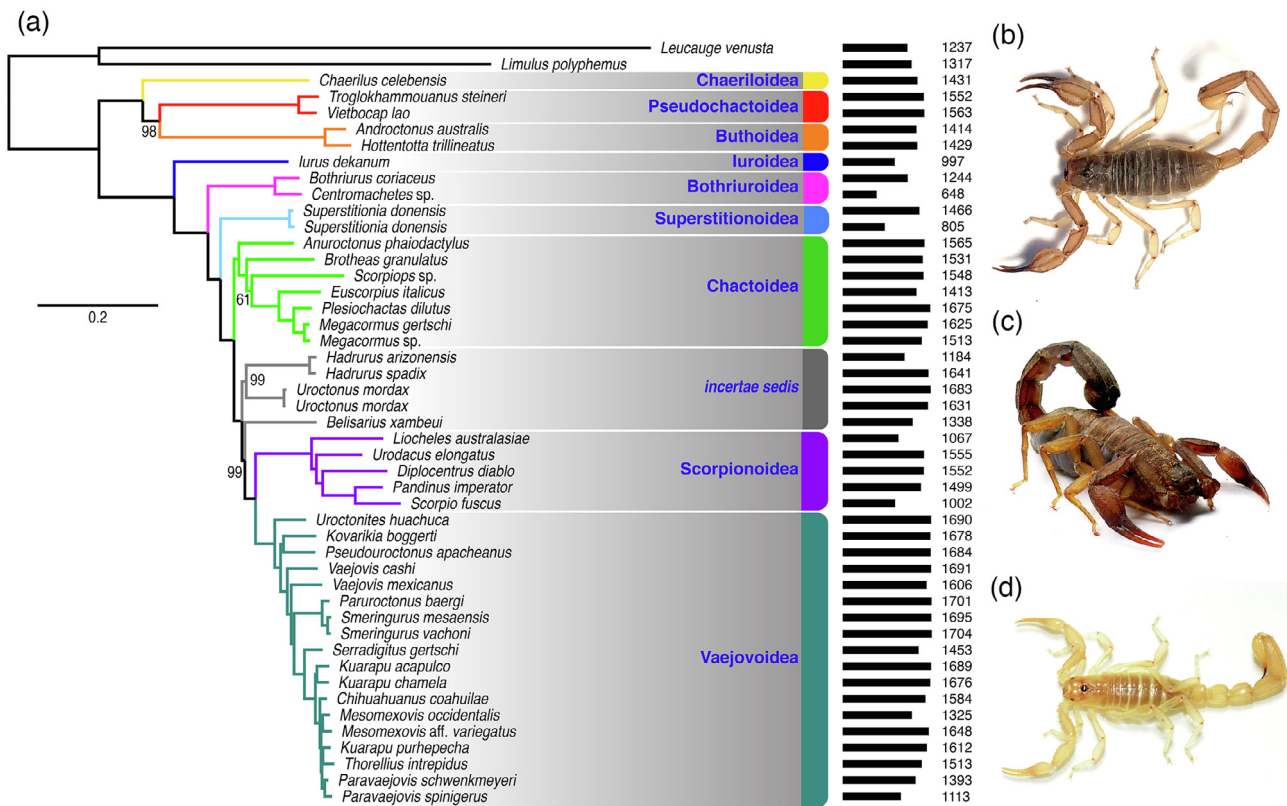


Fig. 2. (a) Maximum likelihood tree topology recovered from the analysis of 1730 orthogroups (Matrix 2; lnL = −13,286,834.8116). Nodes with maximal bootstrap support are shown with black circles. Bars to the right of terminal names indicate the number of loci representing that species. Right column shows live habitus of representative scorpion species from the three subfamilies of the superfamily Vaejovioidea: (b) adult male *Smeringurus vachoni* (Smeringurinae); (c) adult female *Vaejovis mexicanus* (Vaejovinae); and (d) adult male *Paravaejovis schwenkmeyeri* (Syntropinae). Photographs in (b) and (d) by C. Santibáñez-López, (c) by D. Barrales.

4. Discussion

4.1. Detection and resolution of contamination in phylotranscriptomic datasets reveals the phylogenetic position of Vaejovioidea

Since the first phylogenomic analysis of Scorpiones (i.e. Sharma et al., 2015), a major concern weighing heavily upon Vaejovioidea was its recovery as a diphyletic entity. The representation of only two exemplars in that phylogenomic study limited the scope of its conclusions, but the notion of a polyphyletic Vaejovioidea did not accord with the robust support for this family's monophyly in morphological cladistic analyses (Fig. 1). To test the possibility of a contamination, we analyzed other libraries of *Superstitionia donensis* (Santibáñez-López et al., 2016), *Smeringurus mesaensis*, the congeneric species *Smeringurus vachoni*, a library of *Paravaejovis schwenkmeyeri* (Cid-Urbe et al., 2018), and *Serradigitus gertschi* (Romero-Gutiérrez et al., 2018). In addition, to ensure on-target sequencing of the phylogenetically significant terminal *Uroctonus mordax*, this species too was sequenced in different laboratories using different specimens. Our analyses, which leveraged gene trees and gene tree reconciliation approaches (Supplementary Figs. 1–2), confirmed our suspicions that the 2015 library of *S. mesaensis* was contaminated, and its phylogenetic placement recovered in previous analyses (Sharma et al., 2015, 2018) is spurious.

After removal of the contaminated library, we reassessed the phylogenomic relationships of Vaejovioidea. Intriguingly, our analyses again recovered Vaejovioidea as diphyletic, this time due to the placement of *Uroctonus mordax*. Based on morphological characters derived from the hemispermaphore (Monod et al., 2017) and trichobothrial patterns (Gertsch and Soleglad, 1972; Sissom, 1990; Stockwell, 1992; Soleglad and Fet, 2003, 2004; Prendini and Wheeler, 2005), *Uroctonus* has been associated with both Vaejovioidea and Chactidae. All our analyses

recovered *Uroctonus* as neither a member of Chactidae (sensu Soleglad and Fet, 2003, 2004) nor as part of Vaejovioidea (sensu Prendini and Wheeler, 2005). Rather, *Uroctonus* formed a cluster with *Belisarius* and *Hadrurus* with unstable relationships between this trio of genera. This grouping has not previously been recovered by morphological analyses.

By contrast, all non-uroctonine Vaejovioidea consistently formed a clade that was sister group to Scorpionioidea across all analyses. Our results refute the notion of a monophyletic Vaejovioidea as currently defined, as well as the traditional placements of this family as part of Chactoidea and closely related to Chactidae (Fig. 1). The basis for this previously widely held hypothesis stemmed from the broad similarity between Chactidae and Vaejovioidea; Sissom (1990) noted that no morphological characters clearly distinguished these two families. Subsequently, Soleglad and Fet (2003) identified a series of characters putatively uniting the two families, including the presence of two subdistal denticles on the dorsal edge of cheliceral movable finger, the presence of weak to moderate development of a hemispermaphore capsule, and visible genital papillae on males at the posterior edge of their opercula. Members of Vaejovioidea were also distinguished from the rest of the chactoids by the position of the chelal trichobothria *ib-it* on the fixed finger; whereas the rest of the chactoids bore these trichobothria on the palm, with some cases in which they are positioned at the base of the finger (e.g. Soleglad and Fet, 2003). Trichobothrial patterns are a cornerstone of scorpion systematics, but can prove ambiguous for problematic taxa like *Hadrurus* and *Uroctonus* (Vachon, 1974; Stockwell, 1989, 1992; Sissom, 1990; Prendini, 2000; Soleglad and Fet, 2001, 2003). Major “types” of these patterns agree with higher-rank levels of scorpion groups, with some limitations on the lower-rank taxonomic categories. However, trichobothrial patterns remain a workable system to diagnose all levels within Scorpiones until a re-assessment of scorpion morphology is conducted.

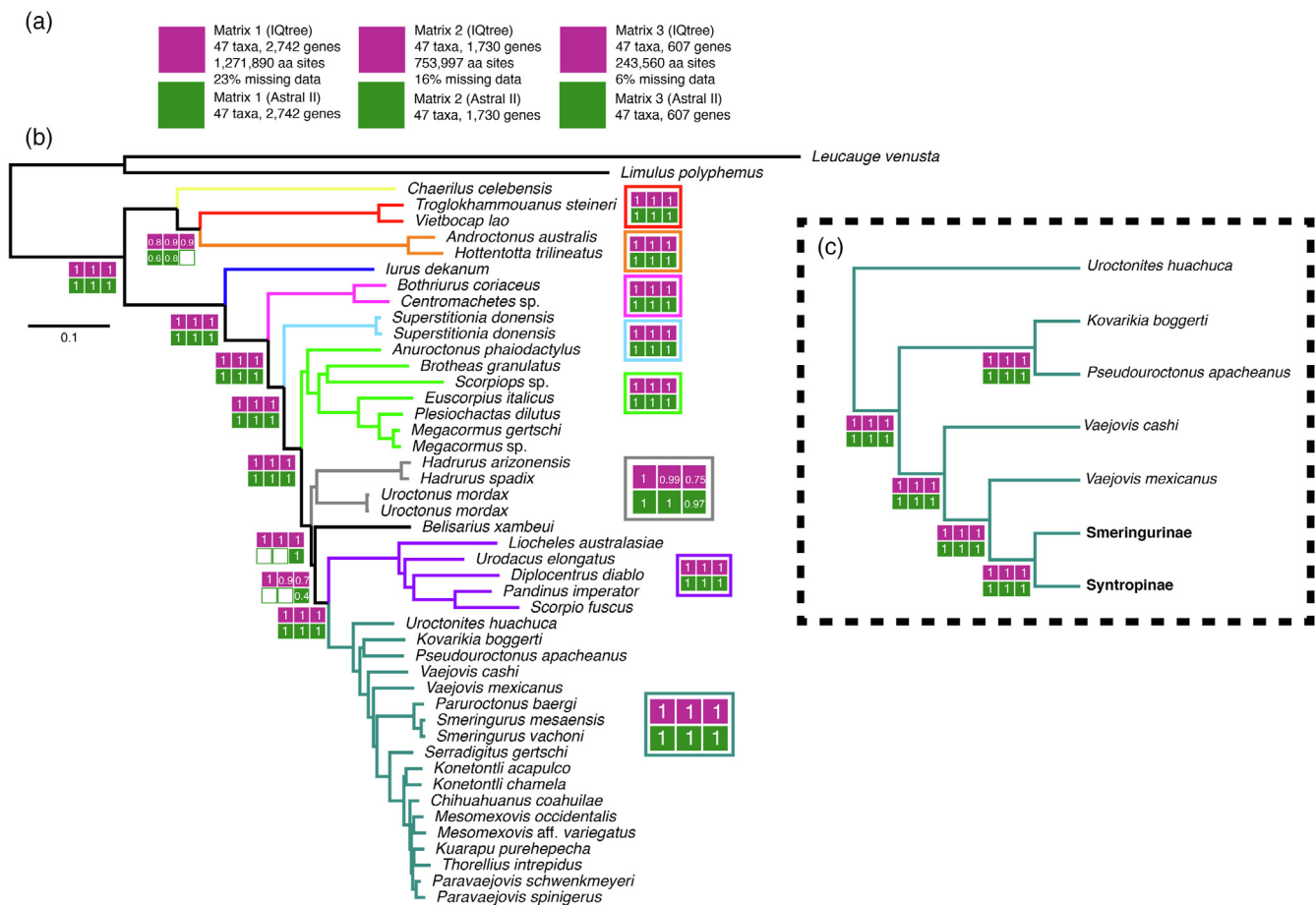


Fig. 3. Phylogenomic sensitive analysis of relationships within Vaejoidea. (a) Supermatrix composition, indicating number of genes, taxa and missing data. (b) Tree topology of Vaejoidea inferred from ML analysis of Matrix 3 (lnL = -4,568,527.6521). Numbers on nodes indicate bootstrap resampling frequencies recovered from the ML analysis of M3. Navajo plots indicate recovery of a given node in the corresponding analysis; numbers in Navajo rug cells indicate bootstrap resampling frequencies recovered in the specific analysis. (c) Nodal support within Vaejoidea.

The tree topology obtained in all analyses indicates a robust relationship between non-uroctonine vaejovids and scorpionoids. This relationship was also never recovered in analyses of morphological characters. However, a recent study on hemispermaphore evolution showed that the number of folds of the bauplan in this structure increases (Monod et al., 2017) in accordance with the phylogenomic topology recovered by Sharma et al. (2015). Thus, the relationship between vaejovids and scorpionids may be consistent with the similarity in the number of folds of the hemispermaphore bauplan (three in Vaejoidea and four in Scorpionoidea).

4.2. Clarifying subfamilial relationships within the non-uroctonine Vaejoidea

Subfamily Syntropinae is the sole member of Vaejoidea whose monophyly has been tested thoroughly using five molecular markers and intensive taxonomic sampling of 145 terminals representing 47 species (González-Santillán and Prendini, 2015b). Here, our results consistently recovered the monophyly of this subfamily, based on analyses of 607–2742 genes. Although, *Serradigitus* was confirmed as sister group of this subfamily, the inclusion of the other *incertae sedis* genera (i.e. *Gertschius*, *Stahnkeus* and *Wernerius*) need to be included in future analyses to support the idea that these genera are also part of this subfamily (González-Santillán and Prendini, 2013). Our results imply that the spinose distal barb margin of the sclerotized hemi-mating plug on the hemispermaphore has evolved only once in Syntropinae (González-Santillán and Prendini, 2013, 2015b). Internal relationships

within this subfamily were partially congruent with the previous topology, although the inclusion of only six of the 11 genera limit our conclusions. Our topologies showed *Konetontli* as the sister taxon to the rest of genera, in agreement with the previous hypothesis. Moreover, our results suggest the monophyly of the *Kochius* clade and reflected a close relationship of *Mesomexovis* and *Chihuahuanus*, and not to *Paravaejovis*.

Previous analyses showed the subfamilial classification within Vaejoidea suffered some flaws (González-Santillán and Prendini, 2015b). Within the problematic subfamilies, Smeringurinae, as defined by Sologlad and Fet (2008), included two tribes (Paravaejovini and Smeringurini) and four genera (*Paravaejovis*, *Paruroctonus*, *Smeringurus* and *Vejovoidus*). In the sole cladistic analysis of Syntropinae (González-Santillán and Prendini, 2015b), Smeringurinae was rendered paraphyletic due to the position of *Paravaejovis pumilis* (species transferred to Syntropinae), but with the position of the other three genera untested. Here, our phylogenomic analyses (using two of the four genera of the tribe Smeringurini *sensu lato*) recovered a clade grouping *Paruroctonus* and *Smeringurus*, in agreement with a previous hypothesis (i.e. Sologlad and Fet, 2008) and retrieved two members of the monophyletic genus *Paravaejovis* as part of the subfamily Syntropinae (González-Santillán and Prendini, 2015b).

The monophyly of subfamily Vaejovinae has not been tested using molecular data. Here, our results consistently recovered this subfamily diphylectic due to the position of *Uroctonites*, *Pseudouroctonus*, *Kovarikia* and *Vaejovis cashi* as sister group to the rest of the vaejovids, with the position of *Vaejovis mexicanus* as sister group to Smeringurinae and

Table 4

Revised higher level classification of extant scorpions. Taxa of questionable monophyly are indicated with asterisks. Taxa with their phylogenetic placement not tested using molecular data are indicated with question marks.

Order Scorpiones Koch 1837
Suborder Neoscorpionina Thorell & Lindström 1885
Infraorder Orthosterni Pocock 1911
Parvorder Buthida Sologlad & Fet 2003
Superfamily Buthoidea Koch 1837
Family Buthidae Koch 1837
Superfamily Chaeriloidea Pocock 1893
Family Chaerilidae Pocock 1893
Superfamily Pseudochactoidea Gromov 1998
Family Pseudochactidae Gromov 1998
Parvorder Iurida Sologlad & Fet 2003
Superfamily Bothriuroidea Simon 1880
Family Bothriuridae Simon 1880
Superfamily Chactioidea Pocock 1893 redefined
Family *Chactidae Pocock 1893
Family Euscorpidae Laurie 1896
Family Scorpidae Kraepelin 1905
Superfamily Iuroidea Thorell 1876
Family Iuridae Thorell 1876
Superfamily Superstitionioidea Stahnke 1940 new superfamilial rank
Family Superstitioniidae Stahnke 1940 new superfamilial assignment
Superfamily Scorpionioidea Latreille 1802
Family Diplocentridae Karsch 1880
Family Hemiscorpidae Pocock 1893
Family *Hormuridae Laurie 1896
Family *Scorpionidae Latreille 1802
Family Urodacidae Pocock 1893
Superfamily Vaejovoidea Thorell 1876 revalidated
Subfamily Smeringurinae Sologlad & Fet 2008
Subfamily Syntropinae Kraepelin 1905
Subfamily ?Vaejovinae Thorell 1876
Subfamily ?Stahnkeninae Sologlad & Fet 2008
Incertain sedis
Family Caraboctonidae Kraepelin 1905
Family ?Heteroscorpionidae Kraepelin 1905
Family Troglotayosidae Lourenço 1998
Family ?Typhlochactidae Mitchell 1971
Subfamily Uroctoninae Mello-Leitao 1934

Syntropinae. Therefore, our analyses show that the position of trichobothria *ib-it* in Vaejovinae is plesiomorphic.

4.3. A revised classification of scorpions

To redress the non-monophyly established for several higher-level groups in this body of work, we take the following systematic actions (Table 4).

- (1) The paraphyly of Chactioidea is partially redressed by the elevation of Superstitioniidae to the superfamily Superstitionioidea (**new superfamily**). This superfamily includes one family: Superstitioniidae. Some studies (i.e. [Prendini et al., 2010](#)) suggested a putative relationship between Superstitioniidae, Typhlochactidae and Troglotayosidae. However, the phylogenetic position of the latter two families has not been rigorously tested. The present topology suggest that these families may be only distantly related, given the placement of Belisarius (Troglotayosidae) and Superstitionia.
- (2) The clade of non-uroctonine vaejovids is restored as the superfamily Vaejovoidea (**status revalidated**).
- (3) Within Vaejovoidea, only two subfamilies were monophyletic: Smeringurinae and Syntropinae. Vaejovinae was recovered

paraphyletic and therefore its status remains uncertain, along with the position of the genera *Gertschius*, *Stahnkeus* and *Wernerius* (*incertae sedis*).

- (4) Chactioidea is restricted to Chactidae (paraphyletic, due to the position of *Anuroctonus*), Euscorpidae, Scorpidae. Caraboctonidae, Troglotayosidae, Typhlochactidae, in addition to subfamily Uroctoninae, are considered *incertae sedis* until key taxa are included in future analyses (e.g., *Caraboctonus*, *Calchas*, *Troglotayosicus*, *Typhlochactas*).

5. Conclusions

This work resolves the phylogenetic position and composition of the family Vaejovidae. Toward a stable higher-level classification system of scorpions, we established two new superfamilies to accommodate the results of our phylogenomic analyses that are unambiguously supported and stable. These new taxa redress the incidence of previously paraphyletic groups. The phylogenetic placement of Vaejovoidea as the sister group to Scorpionioidea in the scorpion tree of life is anticipated to guide the selection of outgroup taxa in future studies of these families, including for future inquiries of biogeographic history and the evolution of morphology.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2019.02.021>.

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