



Research

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Author for correspondence:

Prashant P. Sharma
e-mail: psharma@amnh.org

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Phylogenomic resolution of scorpions reveals multilevel discordance with morphological phylogenetic signal

Prashant P. Sharma¹, Rosa Fernández², Lauren A. Esposito³,
Edmundo González-Santillán⁴ and Lionel Monod⁵

¹Division of Invertebrate Zoology, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA

²Department of Organismic and Evolutionary Biology, Harvard University, 26 Oxford Street, Cambridge, MA 02138, USA

³Essig Museum of Entomology, University of California at Berkeley, 130 Mulford Hall, Berkeley, CA 94720, USA

⁴Laboratorio Nacional de Genómica para la Biodiversidad, Centro de Investigaciones y de Estudios Avanzados del Instituto Politécnico Nacional, and Laboratorio de Aracnología, Departamento de Biología Comparada, Facultad de Ciencias, Universidad Nacional Autónoma de México, Coyoacán, C.P. 04510, México DF, México

⁵Département des Arthropodes et d'Entomologie I, Muséum d'Histoire Naturelle de la Ville de Genève, Route de Malagnou 1, Genève 1208, Switzerland

Scorpions represent an iconic lineage of arthropods, historically renowned for their unique bauplan, ancient fossil record and venom potency. Yet, higher level relationships of scorpions, based exclusively on morphology, remain virtually untested, and no multilocus molecular phylogeny has been deployed heretofore towards assessing the basal tree topology. We applied a phylogenomic assessment to resolve scorpion phylogeny, for the first time, to our knowledge, sampling extensive molecular sequence data from all superfamilies and examining basal relationships with up to 5025 genes. Analyses of supermatrices as well as species tree approaches converged upon a robust basal topology of scorpions that is entirely at odds with traditional systematics and controverts previous understanding of scorpion evolutionary history. All analyses unanimously support a single origin of katoikogenic development, a form of parental investment wherein embryos are nurtured by direct connections to the parent's digestive system. Based on the phylogeny obtained herein, we propose the following systematic emendations: Caraboctonidae is transferred to Chactoidea **new superfamilial assignment**; superfamily Bothriuroidea **revalidated** is resurrected and Bothriuridae transferred therein; and Chaerilida and Pseudochactida are synonymized with Buthida **new parvordinal synonymies**.

“All scorpions look generally alike.”

Gary Allan Polis (1990)

1. Introduction

The evolutionary origins of scorpions (order Scorpiones), one of the most recognizable and charismatic groups of arthropods, have long been shrouded in mystery and engulfed by dispute. Scorpions first appeared in the Silurian and are represented extensively in the Palaeozoic fossil record [1–3], with *ca* 2000 described extant species surviving to the present day [4]. The relationship between scorpions and Eurypterida, the extinct group referred to as ‘sea scorpions’, has been a matter of historical debate [5–10]. The placement of scorpions within Arachnida was also considered controversial [9–13], but recent phylogenomic studies have favoured the Arachnoplumonata hypothesis: the sister group relationship of scorpions and tetrapulmonates (i.e. spiders and allied orders) [14,15].

Paralleling the placement of scorpions among the arachnid orders, the internal phylogeny of scorpions has also long been contentious [16–22]. Numerous workers have historically emphasized different morphological characters of scorpions, resulting in variable hypotheses of phylogeny (figure 1),

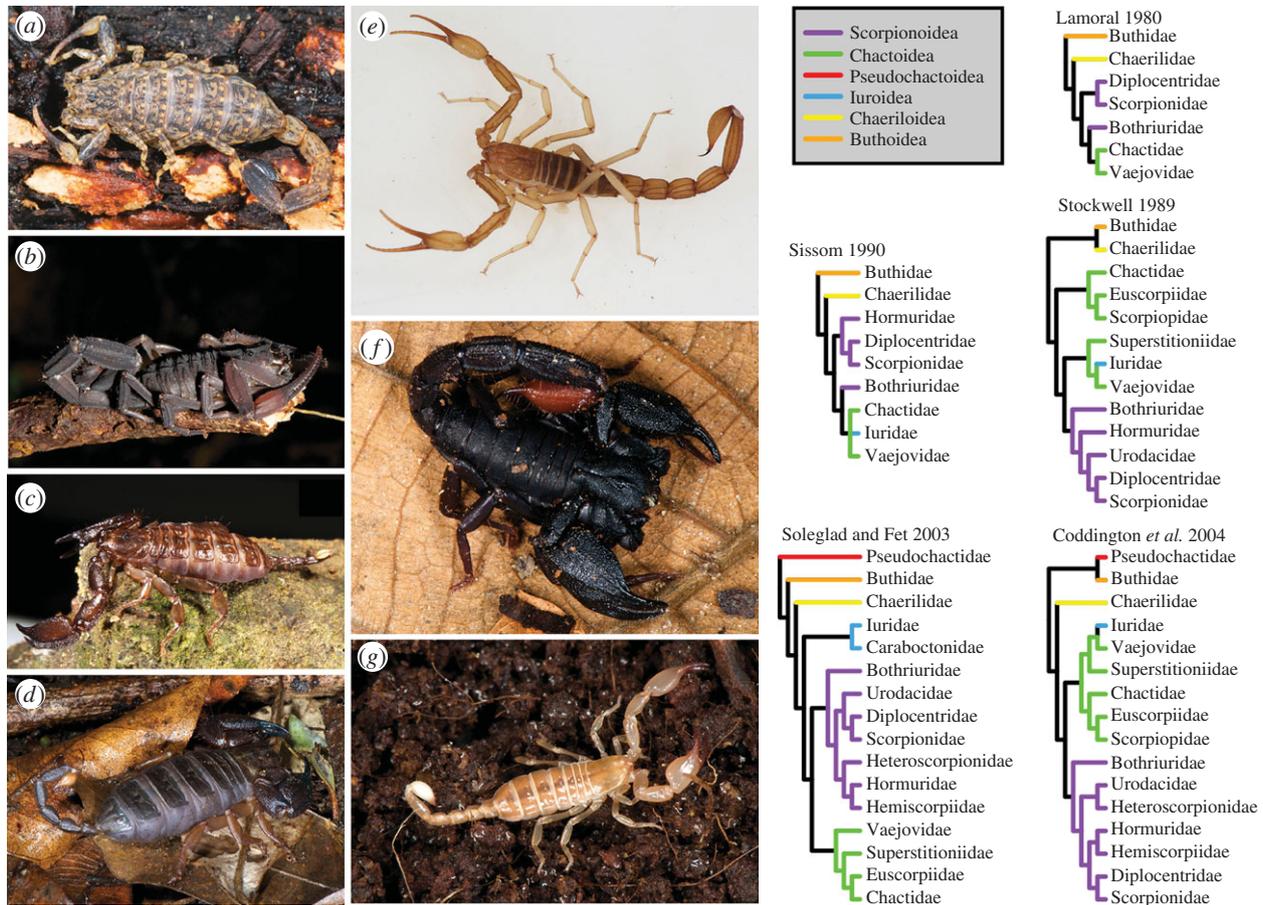


Figure 1. Left: (a–g) exemplars of scorpion diversity. (a) *Isometrus* sp. (Buthidae); (b) *Tityus obscurus* (Buthidae); (c) *Liocheles australasiae* (Hormuridae); (d) *Opisthacanthus* cf. *asper* (Hormuridae); (e) *Vietbocap lao* (Pseudochactidae); (f) *Brotheas granulatus* (Chactidae); (g) *Belisarius xambeui* (Troglotayosicidae). Right: historical hypotheses of scorpion relationships. Colours in tree topologies correspond to superfamilies, as designated by Soleglad & Fet [20]. Photos: G. Giribet. (Online version in colour.)

none of which has been generally accepted [21,22]. Traditionally, scorpions are divided into two morphologically distinct groups, the family Buthidae and the non-buthids [16,19,23]. The phylogenetic position of the relictual families Chaerilidae and Pseudochactidae, which share characters with both groups, has been particularly debated [16,19,23,24].

All previous inferences of scorpion higher level relationships (and ensuing disputes) have been grounded in morphological characters, whose use may be limited in a group that exemplifies morphological stasis [22,24]. Despite widespread use of molecular sequence data for phylogenetic reconstruction, a scorpion phylogeny based on molecular data has yet to be proposed. Use of molecular sequence data from one to three loci is currently limited to analyses of individual families [25,26], or a subset of families in the absence of outgroup taxa [20]. The lack of a reliable phylogeny for scorpions is a major impediment for evolutionary studies of morphology and genomics. For instance, despite significant research efforts on scorpion venoms by means of estimating species trees (EST), transcriptomic or genomic sequencing approaches, conclusions pertaining to the evolution of toxins remain tenuous due to an unknown underlying species tree (e.g. [27–29]). This also holds for investigations of various morphological characters [30–32].

The high gene content exhibited by the first sequenced scorpion genome is suggestive of extensive gene family turnover and duplication events [28], a discovery paralleled by evidence of retention and neofunctionalization of various paralogues early in scorpion evolution [33]. In order to leverage the high gene content of scorpion genomes for testing

phylogenetic relationships, we sequenced full, strand-specific transcriptomes from all extant scorpion superfamilies. We present here, to the best of our knowledge, the first complete higher level molecular phylogeny of scorpions.

2. Material and methods

Methods are described in greater detail with full references in the electronic supplementary material.

(a) Species sampling and molecular techniques

Paired-end (150 bp) transcriptomes were generated for 25 scorpion and one pseudoscorpion species. Additional datasets used as outgroups were obtained from a previous study [15] or from GenBank. Collecting locality information, statistics on sequencing yields and accession numbers are provided as the electronic supplementary material, table S1. All extant families of scorpions were sampled, except for Hemiscorpiidae and Heteroscorpionidae (previously considered sister to, or part of, Hormuridae and Urodacidae, respectively [20,34]), and Typhlochactidae (considered part of or sister to Superstitioniidae; [20,35]). Tissue preservation and RNA sequencing are as described by Sharma *et al.* [15]. All sequenced libraries are accessioned in the Sequence Read Archive. Other materials (described below) are deposited in the Dryad Digital Repository (doi:10.5061/dryad.n0qr5).

(b) Sequence assembly and orthology assignment

Quality filtering, trimming of reads and strand-specific transcriptomic assemblies were conducted as described in the electronic

supplementary material. Predicted open reading frames (ORFs) were assigned to orthologous groups using the Orthologous MAtRix (OMA) algorithm, (OMA stand-alone v.0.99u; [36,37], which has been shown to outperform alternative approaches towards identification of true orthologues and to minimize type I error in orthology assignment [38]. Additional scorpion taxa not sequenced by us (electronic supplementary material, table S1) were obtained from GenBank. For Sanger-sequenced EST and 454 libraries, redundancy reduction was done with CD-HIT as described in the electronic supplementary material. Owing to the small size of additional datasets and/or the quality of the genome of *Mesobuthus martensii*, predicted ORFs were assigned to orthologous groups using OMA in two separate runs, one for Buthidae (*Iurus*, *Chaerilus* and the pseudochactids used as outgroups; taxon occupancy criterion set to representation in at least 19 taxa) and a second for 'Chactoidea' and Scorpionoidea *sensu stricto* (*Iurus* and *Bothriurus* used as outgroups; taxon occupancy criterion set to representation in at least 16 taxa). This was done for computational expediency as well as to ensure representation of the smallest libraries in supermatrices.

(c) Phylogenomic analyses of supermatrices

In order to discern the potential effects of several confounding factors in phylogenomic reconstruction, several supermatrices were constructed: (i) according to gene occupancy (Matrices 1–4), (ii) retaining only orthogroups with demonstrable compositional homogeneity (Matrix 5), (iii) with algorithmic matrix reduction ([39], see also [40]; <http://mare.zfmk.de>) (Matrices 6–7), (iv) by tertiles of per cent pairwise similarity (Matrices 8–10), and (v) by retaining only verified single-copy orthologues common to Arthropoda (Matrices 11–14).

In order to explore the trade-off between number of genes and matrix completeness, four supermatrices were constructed by varying gene occupancy threshold (Matrices 1–4 containing 136, 599, 1557 and 5025 genes, respectively; figure 2*a*). Alignment and masking of ambiguously aligned positions were conducted as described in the electronic supplementary material.

To assess compositional heterogeneity, we analysed each orthogroup in the 1557 gene dataset using BACoCA v. 1.1 [41]. A supermatrix was constructed by retaining only the 131 most compositionally homogeneous orthogroups, defined as having a relative composition frequency variability value below 0.05. Biases stemming from compositional heterogeneity were thus minimized in this 131 gene supermatrix (Matrix 5).

To implement an algorithmic approach to matrix reduction, we used the MAtRix REduction (MARE) method, which estimates informativeness of every orthogroup based on weighted geometry quartette mapping [39]. Reduction of the 599 gene matrix (Matrix 2) resulted in the retention of 453 orthogroups (Matrix 6), and reduction of the 5025 gene matrix (Matrix 4) in the retention of 2580 orthogroups (Matrix 7).

To assess the possibility that evolutionary rate may be conflated with phylogenetic signal, we created three additional supermatrices by dividing orthogroups of the 1557 gene supermatrix (chosen for balance between dataset size and matrix occupancy) approximately into tertiles, using per cent pairwise identity as a proxy for evolutionary rate (Matrix 8 containing the 500 slowest evolving genes, Matrix 9 the 500 genes of intermediate rate and Matrix 10 the 557 fastest evolving genes). In addition, we trialled tree topologies from supermatrices upon culling fast-evolving sites using TIGER v. 1.02 [42], but we observed major loss of phylogenetic signal upon removing sites ranked in one or more of the fastest evolving bins (of 10 equally sized bins), yielding a basal polytomy for two different matrices and the non-monophyly of scorpions. Those analyses are not included in this study, but are available upon request.

To assess the possibility of incorrect topologies stemming from inadvertent inclusion of paralogues, we identified in Matrices 1–4 all tick (*Ixodes scapularis*) orthologues that were found to occur in single-copy across Arthropoda, as identified in the BUSCO-Ar database of OrthoDB [43]. The intersection of BUSCO-Ar orthologues and Matrices 1–4 constituted the basis for Matrices 11–14, respectively (figure 2*a*).

To account for rate heterogeneity, particularly in pseudoscorpion outgroups [15], analyses of all supermatrices incorporated mixture models (CAT + LG4XF or CAT + GTR; [44,45]) in both maximum-likelihood (ML) and Bayesian inference (BI) analyses, as detailed in the electronic supplementary material. RAXML v. 7.7.5 [46] and PHYLOBAYES MPI v. 1.4f [47] were used for ML and BI analyses, respectively.

To account for heterotachy, we implemented ML analyses with mixed branch length models [48] using PhyML + M3L [48–50] for our most complete matrices (Matrix 1 and Matrix 11; sequence occupancy more than 90%), with four branch length mixtures. Heuristic details are provided in the electronic supplementary material.

(d) Phylogenomic analyses of gene trees

As concatenation methods can mask phylogenetic conflict when strong gene tree incongruence is incident, we conducted species tree approaches on best-scoring ML gene tree topologies of constituent orthogroups of the most complete datasets, Matrix 1 (136 genes; 93.0% occupancy) and Matrix 2 (599 genes; 86.9% occupancy). To examine incongruence of constituent genes, we inferred best-scoring ML gene trees for all orthogroups included in these supermatrices. Species trees were estimated from partial gene trees using three partially parametric methods: STAR [51], MP-EST [52] and NJst [53].

To quantify levels of gene tree incongruence, we calculated for every node in Matrices 3 and 4, the available number of potentially informative gene trees (i.e. trees containing at least one member of each descendant branch and two distinct outgroups) and the number of gene trees congruent with those nodes [54]. We mapped these quantities both for the concatenated ML topology recovered by Matrix 3, as well as for alternative topological hypotheses corresponding to traditional systematic relationships.

Following a supernet approach, gene trees were decomposed into quartettes using SUPERQ v. 1.1 [55], and a supernet assigning edge lengths based on quartette frequencies was inferred selecting the 'balanced' edge-weight optimization function.

3. Results

(a) Supermatrix approaches

Our analyses of multiple data matrices (figure 2*a*) result in a well-supported tree topology of scorpions that is greatly incongruent with traditional morphological hypotheses. All analyses yielded a grouping of Buthidae with Chaerilidae (the putative sister group of the remaining Iurida (iuroids, scorpionoids and chactoids) [19,20,23]) and Pseudochactidae (the putative sister group of all other scorpions [20]) (figure 2*b,c*). To facilitate discourse, we henceforth refer to this trio of families as the revised Buthida and synonymize parvorders Chaerilida and Pseudochactida with Buthida **new synonymies** (table 1). The family Chaerilidae was either recovered as sister group to the clade (Pseudochactidae + Buthidae) (figure 2*d*) or, in the majority of analyses, as sister group to Pseudochactidae (Matrices 2 and 3; figure 2*b*), indicating some discordance at the base of Buthida.

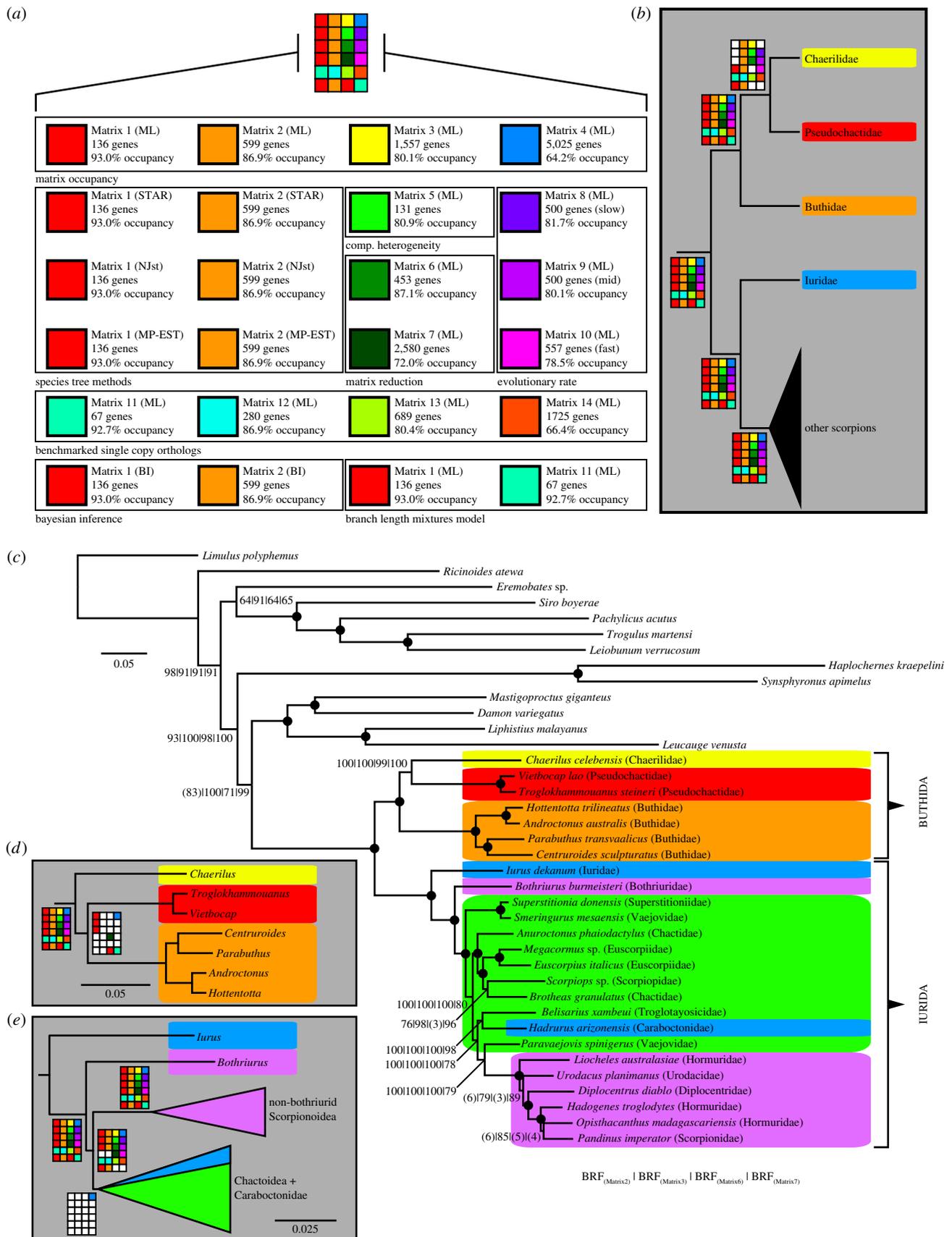


Figure 2. Phylogenomic analyses of scorpions. (a) Supramatrix composition with numbers of genes and gene occupancies, and accompanying methods. (b) Basal topology of Scorpiones. Shaded squares in Navajo plots indicate recovery of a given node in the corresponding analysis. (c) Tree topology of Scorpiones inferred from ML analysis of 1557 gene matrix. Numbers on nodes indicate bootstrap resampling frequencies from ML analyses of Matrices 2, 3, 6 and 7. Black circles indicate maximal nodal support frequencies for all four matrices. (d) Alternative topology at the base of Buthida obtained in seven analyses. (e) Alternative topology within Iurida obtained in one analysis. Shading corresponds to superfamilies (as in figure 1). (Online version in colour.)

Every analysis obtained a basal split between Buthida and Iurida (Iuridae + the remaining scorpions). All analyses refuted the monophyly of Iuroidea (diphyletic), Scorpionoidea

(diphyletic) and Chactoidea (paraphyletic or polyphyletic), as well as the monophyly of three diverse families—Chactidae, Hormuridae and Vaejovidae—with maximal nodal support

Table 1. Revised higher level classification of extant scorpions. (Taxa of questionable monophyly are indicated with asterisks. Taxa of untested phylogenetic placement using molecular sequence data are indicated with question marks.)

Order Scorpiones Koch, 1837
Suborder Neoscorpionina Thorell & Lindström, 1885
Infraorder Orthosterni Pocock, 1911
Parvorder Buthida Soleglad & Fet 2003
Superfamily Buthoidea Koch, 1837
Family Buthidae Koch, 1837
Superfamily Chaeriloidea Pocock, 1893 new parvordinal assignment
Family Chaerilidae Pocock, 1893
Superfamily Pseudochactoidea Gromov, 1998 new parvordinal assignment
Family Pseudochactidae Gromov, 1998
Parvorder Iurida Soleglad & Fet 2003
Superfamily Iuroidea Thorell, 1876
Family Iuridae Thorell, 1876
Superfamily Bothriuroidea Simon, 1880 revalidated
Family Bothriuridae Simon, 1880
Superfamily *Chactoidea Pocock, 1893
Family Caraboctonidae Kraepelin, 1905 new superfamilial assignment
Family *Chactidae Pocock, 1893
Family Euscorpidae Laurie, 1896
Family Scorpipidae Kraepelin, 1905
Family Superstitioniidae Stahnke, 1940
Family Troglotayosicidae Lourenço, 1998
Family [?] Typhlochactidae Mitchell, 1971
Family *Vaejovidae Thorell, 1876
Superfamily Scorpionoidea Latreille, 1802
Family Diplocentridae Karsch, 1880
Family [?] Hemiscorpiidae Pocock, 1893
Family [?] Heteroscorpionidae Kraepelin, 1905
Family *Hormuridae Laurie, 1896
Family *Scorpionidae Latreille, 1802
Family Urodacidae Pocock, 1893

(figure 2*b,c*; electronic supplementary material, figures S1–S20). A single analysis (Matrix 4; 5025 genes) nearly recovered the monophyly of Chactoidea, albeit with a nested inclusion of Caraboctonidae (a member of the superfamily Iuroidea) (figure 2*e*; electronic supplementary material, figure S4). This anomalous tree topology is discussed in detail below.

The placement of *Iurus dekanum* and *Bothriurus burmeisteri* in a grade at the base of the remaining Scorpionoidea and Chactoidea (including Caraboctonidae) was nearly invariable (figure 2*b,e*). For this reason, we transfer Caraboctonidae to Chactoidea **new superfamilial assignment**, and the family Bothriuridae to the resurrected superfamily Bothriuroidea **revalidated** (table 1).

The uniformity of these results indicates that the trade-off between missing data and number of genes analysed does not have a major effect on basal phylogenetic resolution in this case. Accordingly, the distribution of gene representation was nearly uniform for all supermatrices analysed, particularly for ingroup terminals (electronic supplementary material, table S2). The ingroup basal topology yielded by the smaller matrix (Matrix 1) was similar to that of the 1557 gene supermatrix (Matrix 3; electronic supplementary material, figure S3), with minor topological differences among the chactoid lineages and within Buthida.

The basal topology yielded by the 131 gene matrix comprising the most compositionally homogeneous orthogroups (Matrix 5) was identical to that of the 1557 gene supermatrix (electronic supplementary material, figure S7); only a pair of topological differences was observed within the chactoid lineages. An algorithmic approach to maximization of matrix informativeness (MARE) similarly indicated consistency in tree topologies. ML analysis of both reduced supermatrices (Matrices 6 and 7) resulted in the same topology as their respective precursors (Matrices 2 and 4, respectively; figure 2; electronic supplementary material, figures S8 and S9), indicating that discordance at the base of Buthida and elsewhere is not the result of differential gene informativeness in various supermatrices.

With regard to the impact of evolutionary rate on phylogenetic signal, we examined three sub-partitions of a relatively complete matrix (Matrix 3), thereby controlling for the size (i.e. number of sites) and completeness of analysed matrices (note similar dataset occupancies of Matrices 8–10; figure 2*a*). Barring the placement of *Scorpiops* sp. and the outgroup species *Eremobates* sp. in the slowest evolving tertile (Matrix 8, 500 genes), the topologies obtained were identical to the concatenated 1557 gene ML phylogeny (Matrix 3; electronic supplementary material, figures S10–S12). This result contrasts with other studies that have shown a demonstrable effect of evolutionary rate on tree topology [15,56,57], specifically concluding that slowly evolving genes may be more suitable for accurate phylogenetic reconstruction when: (i) long-branch attraction artefacts are incident, and (ii) deep nodes are of interest. But in the present study, we observed relatively uniform patristic distances from the MRCA of Opiliones and Scorpiones upon conducting a Bayesian relative rates test (procedure provided in the electronic supplementary material), suggesting that none of the scorpion species we analysed constitutes a long-branch terminal when inferred with high-occupancy matrices (electronic supplementary material, figure S21). In addition, recent molecular divergence time estimates of arachnids suggest that the diversification of scorpions may not constitute deep (i.e. Palaeozoic) nodes, contrary to previous conjecture [58].

As an external test of OMA's accuracy in predicting single-copy orthologues, the subset of orthologues in Matrices 1–4 that overlapped with benchmarked 'universal' single-copy orthologues common to all arthropods was used to construct another family of supermatrices (Matrices 11–14). These similarly yielded the same basal topology as the majority of analyses, even when the number of constituent orthologues was as low as 67 genes (figure 2; electronic supplementary material, figures S15–S18).

Analyses with mixed branch length models of heterotachy recovered a single branch length category for both Matrices 1 and 11. Basal scorpion relationships (i.e. recovery

of Buthida, Iurida, etc.) were not affected by the use of the branch length mixture models, suggesting that heterotachy does not strongly affect our dataset (figure 2*b*; electronic supplementary material, figures S19 and S20).

(b) Species tree and supernetwork approaches

All three semi-parametric species tree methods (MP-EST, STAR and NJst; see Material and methods) applied to genes included in Matrices 1 and 2 recovered the same basal split between the Buthida and Iurida (figure 2*b*; electronic supplementary material, figures S5 and S6). The STAR and NJst trees derived from gene trees of Matrix 1 recovered the sister relationship of Buthidae and Pseudochactidae (as in the concatenated ML analysis of Matrix 1), but the MP-EST method applied to the same dataset recovered the sister relationship of Chaerilidae and Pseudochactidae (as in the concatenated BI analysis of Matrix 1). These data indicate some incongruence at the base of Buthida. Inversely, the MP-EST method applied to both datasets recovered a more nested placement of *Bothriurus* with respect to all other analyses, indicating additional incongruence within 'Chactoidea' (including Caraboctonidae).

Upon quantifying gene tree incongruence, we observed large numbers of potentially informative genes for almost every node in Matrix 3 (more than 1000 potentially informative genes per node) and Matrix 4 (more than 2000 potentially informative genes per node) (electronic supplementary material, figures S22 and S23). The fraction of potentially informative genes that was congruent with a given node was generally high for both datasets, except for nodes corresponding to divergences within 'Chactoidea'. Intriguingly, similar proportions were obtained for the sister group of Pseudochactidae in Matrix 3 (sister to Buthidae: 0.240; sister to Chaerilidae: 0.230) and Matrix 4 (sister to Buthidae: 0.286; sister to Chaerilidae: 0.250), corroborating incongruence at the base of Buthida. Only a small proportion of gene trees was congruent with the alternative placement of Pseudochactidae at the base of the scorpion tree or the placement of Chaerilidae at the base of Iurida in either dataset (less than 0.15). Similarly, the traditionally held monophyly of Iuroidea, Scorpionoidea (including Bothriuridae), and several families was not supported.

All supernetworks (corresponding to Matrices 2–4) indicate consistency with the major result of the supermatrix and species tree approaches, with largely tree-like networks that bear reticulations (indicative of gene conflict) at the base of Buthida and within Iurida (the nodes corresponding to the base of 'Chactoidea') (electronic supplementary material, figure S24). Comparatively less gene tree incongruence is observed than in other arthropod datasets (e.g. [15,54]).

4. Discussion

(a) A robust hypothesis of scorpion relationships

This study comprises, to our knowledge, the first comprehensive treatment of scorpion phylogenetic relationships with molecular sequence data sampling all major lineages (superfamilies, *sensu* [20]). All analyses converged upon a basal tree topology of scorpions that is greatly at odds with traditional hypotheses based on morphology, at every taxonomic level (figure 2*c*). Unprecedented aspects of our tree topology include the unambiguous inclusion of Buthidae, Chaerilidae and Pseudochactidae in a

clade (parvorder Buthida) sister to the remaining scorpions (parvorder Iurida), controverting the overemphasized significance of plesiomorphic anatomy in Pseudochactidae, or the morphological similarities between Iurida and Chaerilidae [20,24]. The non-monophyly of all superfamilies containing multiple constituent families (Chactoidea, Iuroidea and Scorpionoidea), as well as the non-monophyly of several families represented by multiple terminals, indicates pervasive and strong discordance between traditional systematics and molecular phylogenetic signals.

This result is unusual because morphological characters are selected and defined *a priori* by investigators for their informativeness. In many arthropod clades, clear stepwise gains or losses of morphological characters have historically implied certain basal relationships in groups like insects (e.g. flight; holometaboly), centipedes (e.g. lateral spiracles; number of leg-bearing segments), harvestmen (e.g. direct sperm transfer; median ocelli; paired tarsal claws), and spiders (e.g. unsegmented opisthosoma; venom glands; labidognathous chelicerae), and these evolutionary trends have been robustly validated by phylogenomic data [14,54,59–64]. In the case of scorpions, barring the clear separation of buthids from non-buthid lineages, there has been little agreement as to how scorpion families are related.

The tree topology we obtained across all analyses indicates that most of the character systems commonly used in scorpion systematics are uninformative at the superfamilial level, due to autapomorphic character state distributions with respect to superfamilies or families (electronic supplementary material, figure S25 and table S3) [19,24,65]. A handful of characters unites Iurida and supports the mutual monophyly of Buthidae, Chaerilidae and Pseudochactidae. Almost no characters support interfamilial relationships within Buthida or Iurida, or simply conflict with one another (e.g. within Buthida: cheliceral dentition; hemispermatophore structure; lamellar surface of book lungs). Others still that are variable within these two clades demonstrate homoplasy with respect to the molecular topology (electronic supplementary material, figure S25 and table S3). Our results therefore indicate a need for statistical evaluation of informative discrete morphological character systems (*sensu* [66]), as well as reassessment of palaeontological systematics of the group [1–3].

(b) A single origin of katoikogenic development

Convergent evolution induced by adaptations to substrate type is prevalent in many lineages of scorpions, possibly driving homoplasy in many characters drawn from external morphology [67,68]. Internal morphology may be less prone to homoplasy that stems from adaptation to substrate, and thus may be more informative at the higher taxonomic levels, as exemplified by mode of embryonic development (electronic supplementary material, figure S25 and table S3). While all scorpions are viviparous, most have large, yolky eggs, with embryonic development occurring in the oviduct, and embryos surrounded by embryonic membranes (apoikogenic development). Only a handful of lineages bears small eggs with no yolk or embryonic membranes; development of the embryos occurs in modified outgrowths of the ovarium that enable trophic exchange from the adult female's hepatopancreas to the embryos, via the embryonic chelicerae (katoikogenic development). This unique developmental process unites the non-bothriurid Scorpionoidea, which are invariably recovered as a clade nested within Iurida (figure 2). Concordantly,

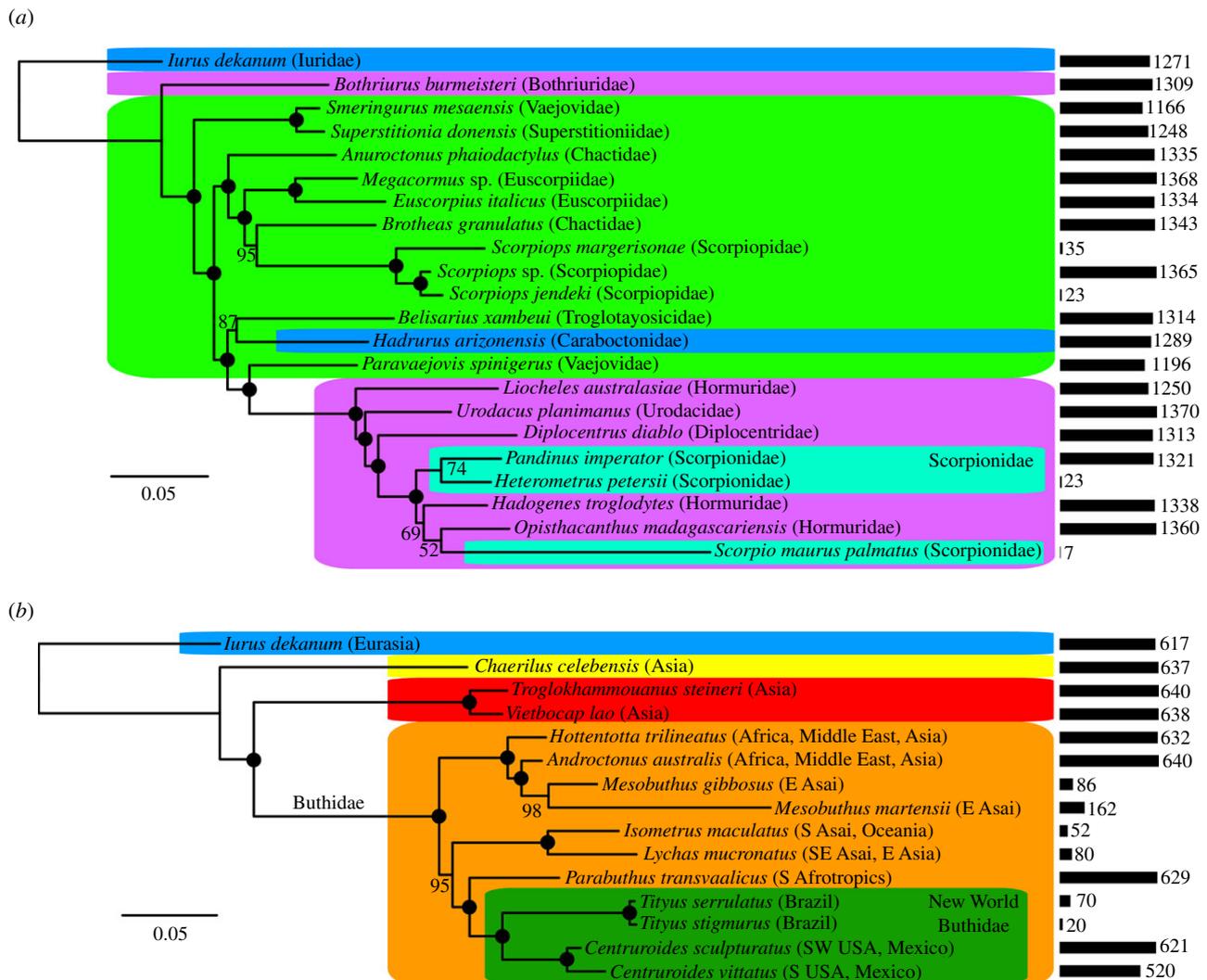


Figure 3. (a) Phylogeny of Lurida based on ML analysis of 1405 genes, including libraries not sequenced in this study. Bars at right with accompanying numbers indicate gene representation for each terminal. Numbers on nodes indicate bootstrap resampling frequencies. (b) Phylogeny of Buthidae based on ML analysis of 649 genes, including libraries not sequenced in this study. Shading corresponds to superfamilies (as in figure 1) and to distinguish Scorpionidae and New World Buthidae. (Online version in colour.)

Bothriuridae, the only putative member of Scorpionoidea that lacks katoikogenic development, was excluded from this clade in all phylogenomic analyses.

The disposition of the digestive glands is distributed in a comparable manner to katoikogenic development (electronic supplementary material, figure S25). All scorpions bear compact digestive glands, excepting the non-bothriurid scorpionoids, which bear digitiform digestive glands [65]. However, the two characters are strongly correlated, probably owing to the physiological and/or physical requirements of katoikogenic development.

(c) Increments to taxonomic sampling reveal additional non-monophyletic groups

The limited taxonomic sampling in this study precludes rigorous investigation of derived relationships, although our analyses surprisingly did suggest the non-monophyly of some families (e.g. Hormuridae, Vaejovidae). Owing to the paucity of genomic resources available for Lurida, few existing datasets can presently be added to our supermatrices, and at considerable expense of matrix occupancy (figure 3; electronic supplementary material, table S2). Adding four small datasets

to our analyses (*Scorpio maurus palmatus*, *Heterometrus petersii* and two species of *Scorpiops*) indicated the monophyly of the genus *Scorpiops* with maximal nodal support, even though few genes (23–35) are available for the *Scorpiops* species; other relationships within ‘Chactioidea’ were not affected (figure 3a). By contrast, addition of two scorpionids rendered the family Scorpionidae diphylectic in the best-scoring ML topology, with *Scorpio maurus palmatus* (represented by seven genes) nesting within the non-*Liocheles* hormurids (bootstrap resampling frequency of 69%; figure 3a).

The paucity of gene representation for the genera *Heterometrus* and *Scorpio* from our dataset, together with the absence of the fourth scorpionid genus, *Opisthophthalmus*, renders the diphyly of Scorpionidae dubious at present. But given robustly supported non-monophyly of Hormuridae and Scorpionoidea *sensu lato*, the monophyly of Scorpionidae must now be regarded with guarded skepticism as well.

Comparatively more genomic resources are available for Buthidae, the most species-rich family of scorpions, which includes nearly all medically significant species. Inclusion of the genome of the buthid *M. martensii* in tandem with several smaller datasets previously sequenced revealed a robust internal phylogeny of available Buthidae (figure 3b), with the

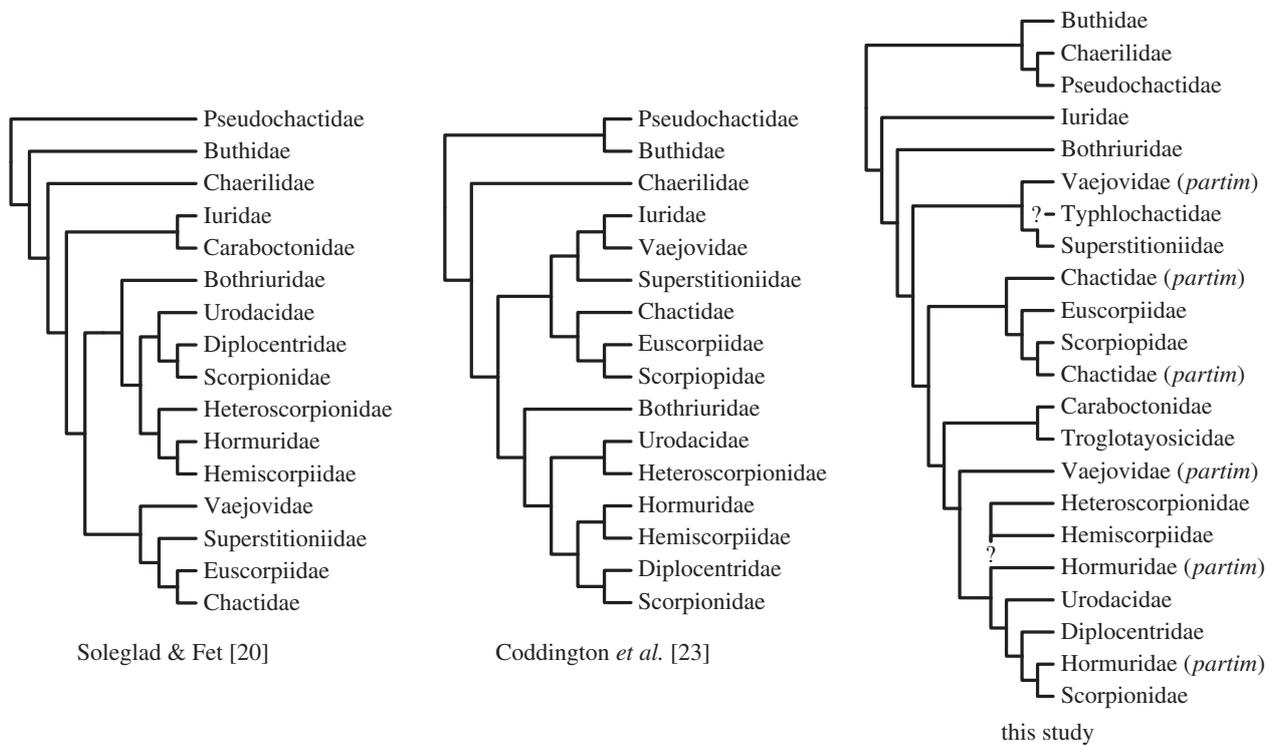


Figure 4. Comparison of historical hypotheses of scorpion relationships (left and middle) with a majority rule consensus all tree topologies obtained in this study. Note non-monophyly of multiple families. Question marks indicate three non-sampled lineages whose putative placement (based on morphology) is now also suspect.

New World buthids (represented here by *Centruroides* and *Tityus*) definitively nested within Old World counterparts. This result is somewhat consistent with an earlier inference based on analysis of 16S rRNA sequences [25]; both results controvert the previously hypothesized basal split between Palaeotropical and Neotropical buthids [23].

(d) Topological incongruence of sparse supermatrices is attributable to non-random distribution of missing data

While the supermatrices we analysed constitute simultaneously some of the largest and most complete in arthropod phylogenomic literature [15,59,60], we obtained the aberrant result of a single analysis somewhat consistent with previous morphology-based systematics: the ML analysis of the 5025 gene supermatrix (figure 2e; electronic supplementary material, figure S4). The tree topology recovered by this analysis yields the monophyly of 'Chactoidea' (including Caraboctonidae, previously in superfamily Iuroidea) and renders Scorpionoidea paraphyletic instead of polyphyletic.

A corollary of its size, the distinguishing feature of Matrix 4 is its amount of missing data, which exceeds that of all other supermatrices we analysed. While this matrix still contains a formidable degree of completeness (64.2% occupancy), the pernicious effects of missing data have been previously elucidated by Roure *et al.* [69], among others. Deleterious and misleading effects of missing data in phylogenomic analyses include model misspecification and exacerbation of long-branch artefacts [69], wherefore nearly all recent phylogenomic studies have emphasized maximizing matrix occupancy (e.g. [59–64]). We therefore focused on identifying whether clade-specific absences of data (i.e. non-random

distribution of missing cells in the matrix) could be driving support for spurious nodes.

Using a permutation-based approach to identify genes with non-random distribution of absences and presences (procedure provided in the electronic supplementary material, Methods section and figure S26a,b; see also [60]), we observed that the number of genes for which missing data distribution is significantly different from random (at $\alpha = 0.05$) increases disproportionately as the taxon occupancy threshold decreases. Whereas the proportion of constituent genes with non-random distribution of missing data is less than 10% for Matrices 1–3, this proportion increases to 20.2% for the 5025 gene matrix (electronic supplementary material, figure S26c). To test whether non-random distribution of missing data contributed to support for spurious nodes recovered by Matrix 4, we ran a separate ML analysis of only the 4008 genes wherein missing data were randomly distributed. In this analysis, we discovered that nodes corresponding to basal relationships within a putatively monophyletic 'Chactoidea' + Caraboctonidae are all unsupported (bootstrap resampling frequencies of 30–36%). The majority of bootstrap replicates support the topology recovered by other analyses, i.e. paraphyly of 'Chactoidea' + Caraboctonidae (figure 2c; electronic supplementary material, figure S26d). These results are consistent with a pervasive effect of non-randomly distributed missing data in sparse supermatrices in inflating nodal support frequencies for spurious relationships [69]. We therefore treat the tree topology of Matrix 4 with scepticism and favour instead the basal tree topology depicted in figure 2, which was robustly supported by all other more complete matrices.

5. Conclusion

We executed multidimensional analyses of some of the largest and most complete datasets in arthropod phylogenomics to

resolve for the first time, to our knowledge, the phylogeny of scorpions, one of the most iconic arthropod groups. In accordance with our results, and to simplify the state of scorpion higher level systematics, we provide herein a revised classification of the group (table 1). The basal topology revealed by our analyses (figure 4), and particularly the placement of Pseudochactidae and Chaerilidae, is anticipated to transform the design of forthcoming studies investigating the early evolution of scorpion venoms, placement of fossils and molecular dating.

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