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A plurality of morphological characters need not equate with phylogenetic accuracy: A rare genomic change refutes the placement of Solifugae and Pseudoscorpiones in Haplocnemata

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Abstract

Recent advances in higher-level invertebrate phylogeny have leveraged shared features of genomic architecture to resolve contentious nodes across the tree of life. Yet, the interordinal relationships within Chelicerata have remained recalcitrant given competing topologies in recent molecular analyses. As such, relationships between topologically unstable orders remain supported primarily by morphological cladistic analyses. Solifugae, one such unstable chelicerate order, has long been thought to be the sister group of Pseudoscorpiones, forming the clade Haplocnemata, on the basis of eight putative morphological synapomorphies. The discovery, however, of a shared whole genome duplication placing Pseudoscorpiones in Arachnopulmonata provides the opportunity for a simple litmus test evaluating the validity of Haplocnemata. Here, we present the first developmental transcriptome of a solifuge (*Titanopuga salinarum*) and survey copy numbers of the homeobox genes for evidence of systemic duplication. We find that over 70% of the identified homeobox genes in *T. salinarum* are retained in a single copy, while representatives of the arachnopulmonates retain orthologs of those genes as two or more copies. Our results refute the placement of Solifugae in Haplocnemata. Subsequent reevaluation of putative interordinal morphological synapomorphies among chelicerates reveals a high incidence of homoplasy, reversals, and inaccurate coding within Haplocnemata and other small clades, as well as Arachnida more broadly, suggesting existing morphological character matrices are insufficient to resolve chelicerate phylogeny.

Guilherme Gainett and Benjamin C. Klementz contributed equally to this study.

KEYWORDS
Arachnopulmonata, genome duplication, paralogy, Pseudoscorpiones, rare genomic changes, Solifugae

1 | INTRODUCTION

In the past two decades, modern invertebrate phylogeny has been broadly rewritten through the lens of molecular sequence data. The scalability of molecular data sets to genomic scales, made possible by ever decreasing costs of molecular sequencing, has facilitated access to densely sampled tree topologies and evaluative comparisons of different classes of molecular markers (Edwards, 2016; Mongiardino Koch, 2021; Rokas & Holland, 2000; Salichos & Rokas, 2014). In tandem, conceptual advances have unlocked investigation of nonhierarchical relationships between genes and species, using coalescent-based tree inference approaches and network representations of evolutionary processes (Blair & Ané, 2019; Degnan, 2018; Hibbins & Hahn, 2022; Jiang et al., 2020). Traditional implementation of morphological data using discretized characters and cladistic morphological analyses have concomitantly declined in predominance (Giribet, 2015; Wanninger, 2015). Yet, many hypotheses persist in the animal tree of life that are primarily or exclusively based on morphological character data, particularly in cases where molecular data have yielded conflicting results, as exemplified by debates over the phylogenetic position of sponges and comb jellies at the base of the animal tree of life (Kapli & Telford, 2020; Presnell et al., 2016; Redmond & McLysaght, 2021; Telford et al., 2016; Whelan & Halanych, 2017; Whelan et al., 2015). Promisingly, recent advances in chromosomal-level genome assembly and computational analyses of genomes have begun offering solutions for resolving contentious nodes in the tree of life, as saliently demonstrated by the plesiomorphic architecture of comb jelly genomes and the discovery of a series of rare genomic changes that place sponges closer to cnidarians and bilaterians as the newly named clade Myriazoa (Schultz et al., 2023).

Yet, despite advances in the scalability, assembly, and analysis of molecular sequence data, assessing the phylogenetic position of many higher-level invertebrate lineages remains intractable. Chelicerata (sea spiders, horseshoe crabs, and terrestrial arachnids) represents one such problematic branch of the tree of life. The phylogenetic relationships between many of the constituent orders remain poorly resolved. This recalcitrance can be attributed, in part, to an ancient origin and subsequent rapid diversification of the extant orders (Dunlop, 2010; Rota-Stabelli et al., 2013), producing short internodes. Likewise, several long-branch orders occur among the chelicerates (Acariformes, Parasitiformes, Pseudoscorpiones, and Palpigradi) demonstrating accelerated rates of evolution and yielding artifactual clustering of such lineages near the base of the tree (Ontano et al., 2022; Sharma et al., 2014a). Chelicerate phylogeny has also been hindered by consistent undersampling of key lineages (e.g., Opilioacariformes; Palpigradi). In fact, relatively few molecular phylogenetic studies have sampled all extant chelicerate orders (Ballesteros et al., 2022; Giribet, 2002; Regier et al., 2010; Sharma et al., 2014a). Among these studies, few lineages demonstrate a stable placement, aside from the sister group relationship of Pycnogonida (sea spiders) and Euchelicerata (horseshoe crabs + terrestrial arachnids), the monophyly of Tetrapulmonata, and more recently, the interordinal relationships among the arachnopulmonates (terrestrial arachnids that ancestrally bore book lungs) (Ballesteros et al., 2022; Ontano et al., 2021).

While discordant tree topologies abound in molecular phylogenetic analyses of chelicerates, so too does discordance arise when comparing topologies produced via analysis of morphological characters and molecular sequence data. For example, Xiphosura (horseshoe crabs) are routinely recovered as the sister group to the terrestrial arachnids in morphological analyses (e.g., Shultz, 1990, 2007; Weygoldt & Paulus, 1979; Wheeler & Hayashi, 1998), based on characters such as presence of gills and presence of appendages on the seventh opisthosomal segment (*contra* terrestrial chelicerates). The position of Xiphosura in relation to Arachnida has long held major implications for arthropod evolution, supporting a single terrestrialization event in the common ancestor of the terrestrial chelicerates. However, the monophyly of Arachnida in molecular analyses is frequently poorly supported due to a nested position of Xiphosura. Regier et al. (2010) recovered arachnid monophyly in only two of four analyses. Sharma et al. (2014a), leveraging a 3644-ortholog data set sampling all but two extant chelicerate orders, recovered a nonmonophyletic Arachnida with maximal nodal support, whereas arachnids were also recovered as monophyletic with maximal nodal support when using a subset of slowly-evolving genes. Sharma et al. (2014a) attributed the nonmonophyletic arachnid topology to long-branch attraction artifacts, given the clustering of the known

long-branch orders Pseudoscorpiones, Acariformes, and Parasitiformes at the base of the arachnids. The authors also recovered a nested position of Xiphosura as sister group to Ricinulei with 100% bootstrap resampling frequency. Ballesteros and Sharma (2019), however, recapitulated a sister relationship to Ricinulei, with 100% bootstrap resampling frequency in partitioned IQ-Tree ML analysis of both 1499 and 3534 loci, 100% posterior probabilities in ASTRAL analysis of the same data sets, as well as 96% bootstrap resampling frequency in partitioned EXAML analysis of the 1499 loci data set. An identical placement is also recovered in Ballesteros, Santibáñez-López et al. (2019) with 96% bootstrap resampling frequency in concatenated ML inference of 179 loci. Finally, Ballesteros et al. (2022) recovered a nested position of Xiphosura across various phylogenomic analyses with site heterogeneous model-based approaches implementing both CAT+GTR+Γ and SR4 recoding. Thus, with evidence increasingly supporting a nonmonophyletic Arachnida, the understanding of chelicerate terrestrialization becomes increasingly complex, requiring either multiple colonizations of land or a reversion to an aquatic habitat in the ancestor of Merostomata (horseshoe crabs, Eurypterida, and allied fossil groups).

Disagreement between morphology and molecules is not limited to the placement of horseshoe crabs. Morphology-based analyses have also recovered numerous other clades unsubstantiated by molecular sequence data. These include Dromopoda (Opiliones + Scorpiones + Pseudoscorpiones + Solifugae) and Stomothecata (Opiliones + Scorpiones) (Shultz, 1990, 2007). Suggested synapomorphies of Dromopoda include a reduced prosomal sternum, bicondylar femoropatellar and patellotibial joints, and transverse carapacal furrows. Stomothecata is principally supported by the presence of the stomotheca, the preoral chamber formed by the labrum and endites of the pedipalps and first pair of walking legs. Yet, molecular analyses have failed to recover any support for Stomothecata or Dromopoda (Ballesteros et al., 2022; Howard et al., 2020; Lozano-Fernandez et al., 2019; Regier et al., 2010).

While many chelicerate lineages show unstable phylogenetic positions across morphological and molecular data sets, the advent of genomic architecture and discovery of rare genomic changes has demonstrated the capacity to resolve contentious nodes. The discovery of a shared whole genome duplication (WGD) in spiders and scorpions, supported by the retention of paralogs of key developmental patterning genes and conserved patterns of embryonic gene expression, provided a complex character supporting the monophyly of Arachnopulmonata (Gainett & Sharma, 2020; Leite et al., 2018; Schwager et al., 2007, 2017; Sharma et al., 2014b). This complex character has already been leveraged to resolve the position of Pseudoscorpiones, one of the several long-branch chelicerate orders. Ontano et al. (2021) generated a high-quality developmental transcriptome and a draft genome for pseudoscorpions, revealing the presence of duplicated copies of many homeobox genes shared with representatives of the arachnopulmonates. These duplicates include nine of the ten Hox genes present in panarthropods, various appendage-patterning genes, and microRNAs. The complexity inherent in a shared genome duplication also makes it a formidable character in phylogenetic analyses. Beyond the counts of duplicated genes and microRNAs, Ontano et al. (2021) also found that gene trees of arachnopulmonate ohnologs (duplicates resulting from WGD) tended to feature duplicated clusters of genes that united pseudoscorpions and the remaining arachnopulmonates, reflecting the shared duplications. In some of these cases, it has previously been shown that the duplicated gene copies retain arachnopulmonate-specific expression patterns (i.e., one copy exhibits one particular expression pattern across arachnopulmonates, and its duplicate exhibits a second expression pattern across arachnopulmonates; Gainett & Sharma, 2020; Gainett et al., 2023; Nolan et al., 2020). The repeated incidence of duplicated genes, with specific gene tree topologies, and with specific expression patterns across arachnopulmonate genomes, make scenarios of independent genome duplication within arachnopulmonates highly non-parsimonious. Based on these data sets, Ontano et al. (2021) resolved the placement of Pseudoscorpiones as sister group to the Scorpiones in the clade Panscorpiones.

Validation of arachnopulmonate monophyly based on shared genomic architecture presents additional opportunities for assessing the placement of other chelicerate orders, particularly those that have historically clustered with members of the arachnopulmonates in morphological analyses. Solifugae (commonly known as camel spiders, sun spiders, or solpugids), present such a case, given their variability in phylogenetic position across molecular analyses (Figure 1a). Regier et al. (2010) recovered a sister relationship of Solifugae and Ricinulei, albeit with low nodal support. Sharma et al. (2014a) recovered Solifugae as part of a grade at the base of Chelicerata with other unstable orders in a maximum likelihood analysis of a 3644 ortholog data set with 100% bootstrap resampling frequency. Concatenation of the data set to include only the 500 slowest-evolving genes, however, recovered a grouping with Ricinulei, with this clade in turn sister to Opiliones, and with near-maximal nodal support. Some version of this result was subsequently obtained, sometimes as (Xiphosura + Ricinulei) + Solifugae, by various workers (Ballesteros & Sharma, 2019;

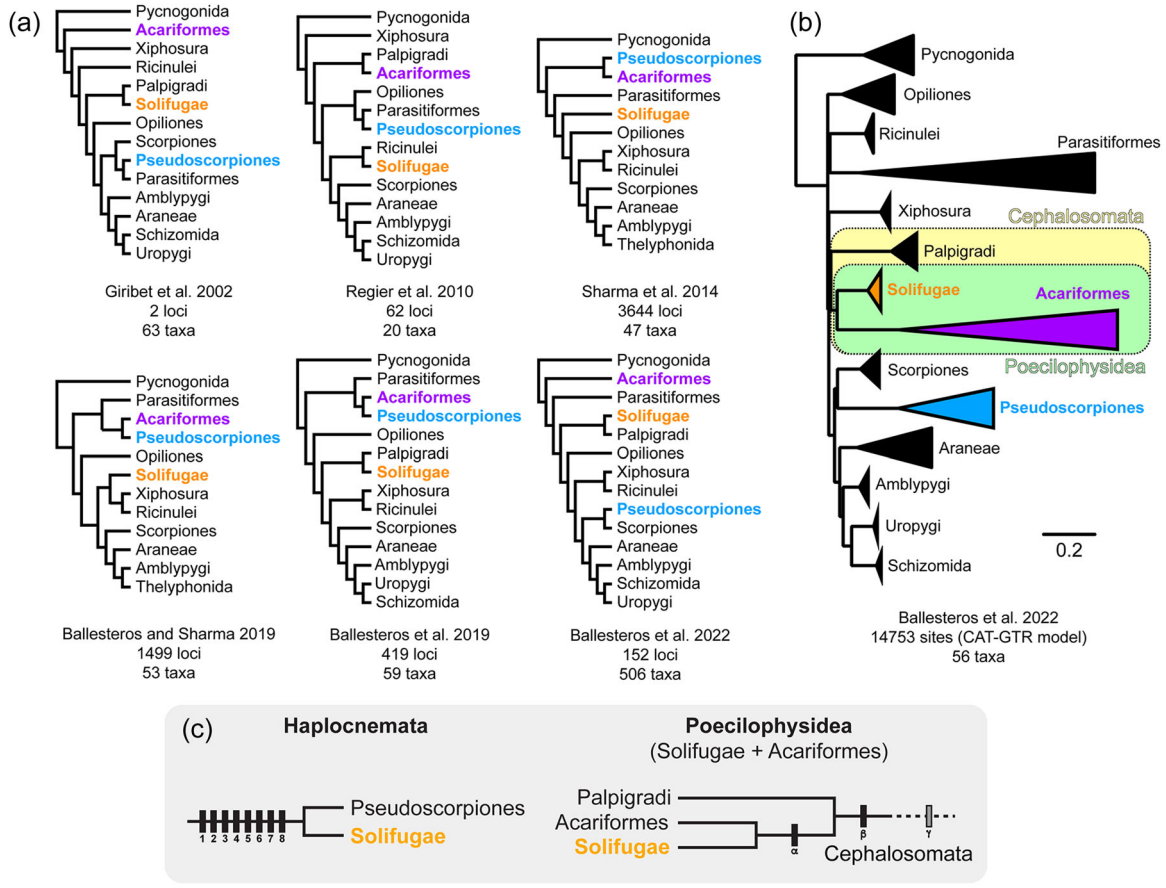

FIGURE 1 (a) Selected tree topologies for the higher-level phylogeny of Chelicerata. Notations below trees indicate number of loci and taxa. Colored taxa correspond to lineages of interest. (b) Summary of chelicerate phylogeny from CAT model-based analyses of Ballesteros et al. (2022). Note the monophyly of Poecilophysidea (Solifugae + Acariformes) and Cephalosomata (Poecilophysidea + Palpigradi). (c) Competing morphological hypotheses for the placement of Solifugae. Left: Haplocnemata (Solifugae + Pseudoscorpiones) is supported by eight putative synapomorphies (Shultz, 2007); character list provided in Table 1. Right: Poecilophysidea and Cephalosomata are each supported by one putative synapomorphy, and these taxa may also be united with other orders by a feature of sperm ultrastructure; character list provided in Table 1. [Color figure can be viewed at wileyonlinelibrary.com]

Ballesteros et al., 2022), though Ballesteros, Santibáñez-López et al. (2019) previously obtained Solifugae + Palpigradi, a grouping supported by the morphology of the coxal gland in these two orders.

In the most comprehensively sampled phylogenomic effort to date, Ballesteros et al. (2022) obtained the intriguing result that filtering genes for low rates of saturation and applying site heterogeneous models (CAT+GTR+Γ and SR4 recoding) recovered the clade Solifugae + Acariformes with high nodal support (Figure 1b). This grouping was previously termed Poecilophysidea by Pickard-Cambridge (1876) and resurrected by Pepato et al. (2010; see also Dunlop, 1999), on the basis of morphological similarities of the prosomal architecture shared by camel spiders and acariform mites (Dunlop et al., 2012). Specifically, only Acariformes and Solifugae possess a ventral division of the prosoma between coxae 2 and 3 known as the sejugal furrow, delineating the propeltidium (the fusion of segments through the second pair of walking legs) from the mesopeltidium (Dunlop et al., 2012). Moreover, the analyses of Ballesteros et al. (2022) also recovered Poecilophysidea as a clade sister group to Palpigradi, a grouping termed Cephalosomata by Pepato et al. (2010) and united by the shared pattern of tagmatization of the anterior seven body segments. Rather than the single, fused dorsal shield spanning the seven prosomal segments (i.e., ocular segment plus six appendage-bearing segments) across other arachnids, the prosomal shield of camel spiders, acariform mites (Bolton, 2022), and palpigrades is divided into propeltidium, mesopeltidium, and metapeltidium, with the latter two representing the free segments of the third and fourth pairs of walking legs, respectively.

Congruence of anatomical characters and molecular topologies is a promising sign of phylogenetic accuracy;

thus, the independent recovery of Poecilophysidea across disparate data types initially appeared to herald a rare moment of consilience in chelicerate phylogeny. However, morphological data sets, rather than supporting any of the above phylogenetic positions, have instead tended to support a sister group relationship of Solifugae with Pseudoscorpiones, forming the clade Haplocnemata (Figure 1c) (Bicknell et al., 2019; Selden et al., 2015; Shultz, 1990, 2007; Weygoldt & Paulus, 1979; Wheeler & Hayashi, 1998). This grouping alternatively appears as Apatellata in the literature, based on the putative absence of the patella in this pair of orders (van der Hammen, 1977). Haplocnemata is supported by eight primarily external morphological characters (Table 1). Three synapomorphies relate to the structure of the chelicerae and include the presence of two segments, ventrolateral intrinsic articulation, and dorsolateral articulation with the carapace. Both Solifugae and Pseudoscorpiones are also suggested to share a rostrosoma, a preoral structure formed by the endites of the pedipalpal coxae and the labrum, as well as a midventral sternapophysis. Together, both structures form a beak-like preoral complex. Additional synapomorphies include the meeting of leg coxae along the ventral midline; elongated, femur-like patellae; and a respiratory system composed of, at least in part, paired tracheal tubules that open as spiracles on opisthosomal segments three and four (Shultz, 2007).

At first glance, Haplocnemata is clearly the more parsimonious phylogenetic position for Solifugae, given the imbalance in number of apparent morphological synapomorphies. Only one character supports each of Poecilophysidea and Cephalosomata (Table 1 and Figure 1c), compared to the eight supporting Haplocnemata. Rarely has the validity of Haplocnemata been called into question in the morphological literature, though Alberti and Peretti (2002) showed that Solifugae, Acariformes, Palpigradi, Parasitiformes, and Opiliones share the condition of aflagellate spermatozoa; Pseudoscorpiones, by contrast, possess sperm with a flagellum coiled around the cell body, a trait they share with Ricinulei and Tetrapulmonata. Alberti and Peretti (2002) argued against the validity of Haplocnemata on this basis and suggested that the suite of characters supporting this clade may be of questionable value—a position refuted by Shultz (2007), who deemed it unlikely that independent character systems could be prone to convergence. While molecular sequence data have consistently failed to recover Haplocnemata—with only total evidence analyses combining a small number of Sanger loci with morphology able to recover this relationship (e.g., Giribet et al., 2002)—phylogenomic analyses have also failed to converge on a stable placement of Solifugae.

Reconciling the dissonance between morphological and sequence-based data sets may benefit from an approach grounded in rare genomic changes. The placement of Pseudoscorpiones within Arachnopulmonata on the basis of a shared WGD in the arachnopulmonate common ancestor offers a simple litmus test of the phylogenetic position for Solifugae. If Solifugae are the sister group of Pseudoscorpiones, it stands to reason that Solifugae must then also share the arachnopulmonate WGD. Alternatively, if Solifugae are more closely related to other apulmonate arachnid orders with aflagellate sperm (e.g., Palpigradi; Acariformes), then they should exhibit an unduplicated genome, comparable to data sets from Acariformes, Parasitiformes, and Opiliones (Gainett et al., 2021; Grbić et al., 2011; Gulia-Nuss et al., 2016; Hoy et al., 2016). To date, such interrogations have not been possible for Solifugae due to

TABLE 1 List of putative synapomorphies underlying competing hypotheses of solifuge placement.

Haplocnemata	Cephalosomata/Poecilophysidea
1. Two-segmented chelicera	α. Sejugal furrow
2. Chelicera with ventrolateral intrinsic articulation	β. Tagmosis of prosoma (pro-, meso-, metapeltidium)
3. Chelicera with dorsolateral carapacal articulation	γ. Aflagellate spermatozoa
4. Rostrosoma	
5. Elongate patellae	
6. Midventral sternapophysis	
7. Coxae meet at ventral midline	
8. Paired spiracles on O3 and O4	

Note: Characters for Haplocnemata based on Shultz (2007); characters for Poecilophysidea/Cephalosomata based on Pepato et al. (2010).

their poorly studied developmental biology and dearth of genomic resources.

Here, we present the first developmental transcriptome of a solifuge obtained from two embryonic stages of the South American ammotrechid *Titanopuga salinarum* Iuri, 2021 (Figure 2). We show that the developmental transcriptome of this solifuge lacks evidence of systemic duplication of developmental patterning genes (contrary to pseudoscorpions), and thus these data for Solifugae refute the Haplocnemata hypothesis.

2 | MATERIALS AND METHODS

2.1 | Solifuge husbandry and collection of embryos

Adult female *T. salinarum* (Figure 2a) were collected in Las Salinas Grandes (64°48′ S, 30°02′ W), Córdoba, Argentina in November 2022. Animals were housed in 38.1 × 25.4 × 20.3 cm plastic containers containing soil from the collection site and a small bottle cap with water. Containers were kept in a dark room at ca. 28°C and 40% humidity. Females were watched for egg clutches weekly. Eight clutches were obtained between November 15 and December 15, 2022, with clutch sizes ranging from 8 to 26 eggs. Viability of clutches was variable, which may be attributable to either some clutches not being fertilized, or that the rearing conditions employed in our study might be suboptimal, as most of the eggs became either dehydrated or moldy. One female (#204) laid a small clutch on December 1, 2022 (Figure 2b), of which five eggs were at a limb bud stage on December 12, 2022 (Figure 2c,d). Embryos of this clutch were first rinsed in 5× phosphate-buffered saline (PBS) and subjected to fixation. Three embryos were transferred to an RNAse-free 1.5 mL tube and frozen at −80°C. The two remaining embryos were transferred into a 3.2% paraformaldehyde solution in 5× PBS and peeled from the vitelline membrane with fine forceps. Embryos were fixed for 20 min and immediately washed in 5× PBS, followed by gradual dehydration into pure ethanol.

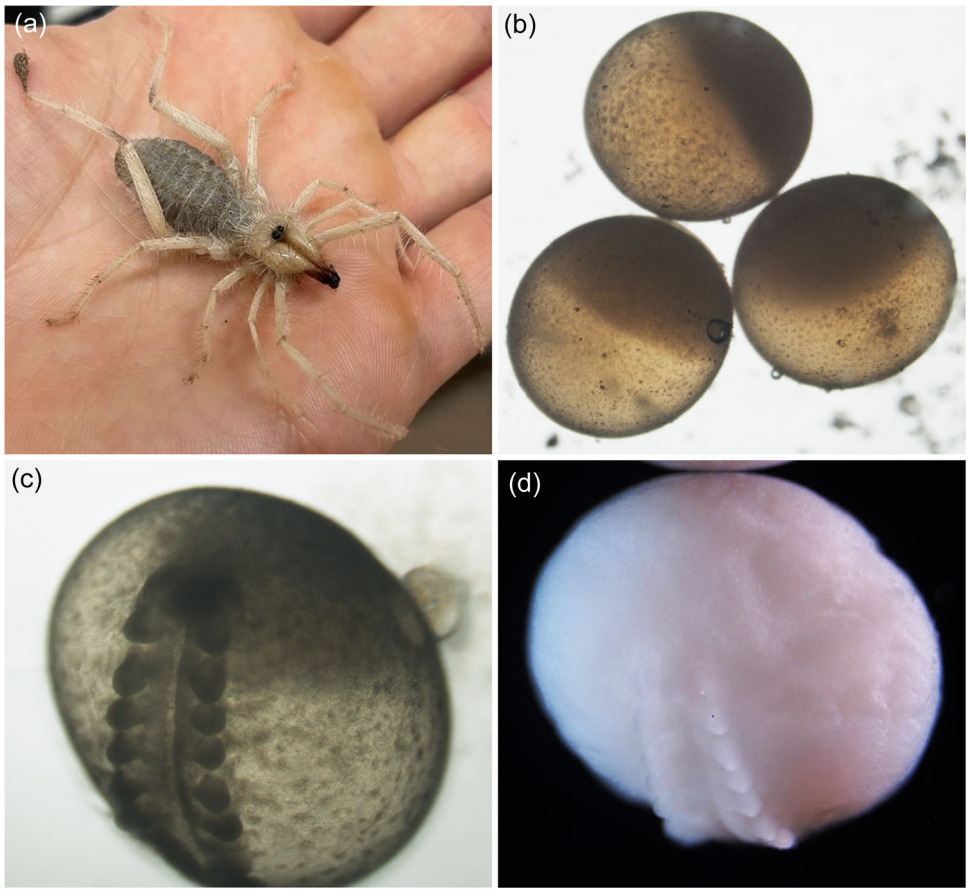

FIGURE 2 (a) Live habitus of *Titanopuga salinarum* (Solifugae, Ammotrechidae), Córdoba, Argentina. (b) Embryos of *T. salinarum* at the germ disc stage, brightfield imaging in phosphate-buffered saline. (c) Embryo of *T. salinarum* at the limb bud stage, brightfield imaging in phosphate-buffered saline. (d) Fixed embryo of *T. salinarum* at the limb bud stage, brightfield imaging in phosphate-buffered saline. [Color figure can be viewed at wileyonlinelibrary.com]

2.2 | RNA sequencing and analyses of homeobox genes

Next-generation sequencing for *T. salinarum* was performed on an Illumina NovaSeq with a 2 × 150 bp paired-end sequencing strategy. Raw reads are deposited in SRA under SRR24782649 (germ disc stage; *T. salinarum*) and SRR24782648 (limb bud stage; *T. salinarum*). De novo assembly was conducted with the software Trinity v.2.15.0 (Grabherr et al., 2011). Inference of transcriptome quality was performed using BUSCO v.5.5. (Simão et al., 2015). Voucher specimens (adult females) were deposited in the arachnology collection of the LABRE, IDEA, Argentina.

Assessment of systemic gene duplications in the solifuge transcriptome focused on the well-studied homeobox gene family, following the data sets and pipeline of Leite et al. (2018) and Ontano et al. (2021). Transcriptomes were queried via tBLASTn searches using a set of 108 homeodomain protein sequences from homeobox genes of the fruit fly *Drosophila melanogaster*, the spider *Parasteatoda tepidariorum*, and the scorpion *Centruroides sculpturatus*. All unique best hits were retrieved, and the longest ORFs were obtained using TransDecoder v5.0.1 (Transdecoder.LongOrfs; minimum length: 50 aa) for subsequent protein prediction (Haas et al., 2013). Protein prediction (Transdecoder.Predict) utilized blastp (BLAST v. 2.10.0) (Altschul et al., 1990) with the UniProt Swiss-Prot database (Bateman et al., 2023), and HMMER v3.2.1 (Wheeler & Eddy, 2013) with the Pfam v. 35.0 database (Finn et al., 2016). Protein sequences were submitted to CDD-search online server (Marchler-Bauer & Bryant, 2004) (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) for domain annotation, and all the sequences containing a homeodomain signature (PSSM 444687) were selected.

For *T. salinarum*, the resulting 104 sequences were subjected to 98% similarity clustering with CD-HIT (Fu et al., 2012; Li & Godzik, 2006) (resulting in 77 sequences) and the longest isoform per Trinity gene was obtained with the Trinity package script "*get_longest_isoform_seq_per_trinity_gene.pl*," resulting in the final 63 transcripts. All solifuge protein sequences were aligned to homeodomain protein sequences from *D. melanogaster*, *P. tepidariorum*, and *C. sculpturatus* with ClustalW (Sievers et al., 2011) and trimmed with GBlocks v.0.91 (Castresana, 2000). Annotation was based on a maximum likelihood phylogenetic analysis conducted with IQ-TREE (Nguyen et al., 2014). Transcripts not sharing unambiguous alignments and placed outside the fly-arachnid clades for each homeobox gene were manually inspected, reciprocally blasted against NCBI database, and further analyzed in individual gene trees from alignments from the data set of Ontano et al. (2021).

2.3 | Hybridization chain reaction

As validation of on-target transcriptomic assembly, probes were designed for homologs of two genes that (1) are duplicated in arachnopulmonates, (2) are single-copy in nonarachnopulmonate arachnids, and (3) have known expression patterns for both arachnopulmonate duplicates and nonarachnopulmonate homologs. For these assays, we selected the Hox gene *Deformed* (expression known for homologs in spiders, scorpion, harvestman, mite, horseshoe crab, and sea spider; Gainett et al., 2023; Jager et al., 2006; Schwager et al., 2007, 2017; Sharma et al., 2012b; Telford & Thomas, 1998) and the limb patterning gene *extradenticle* (expression known for homologs in spiders, scorpion, and harvestman; Nolan et al., 2020; Pechmann & Prpic, 2009; Prpic & Damen, 2004; Sharma et al., 2012a).

Probes for hybridization chain reaction (HCR) gene expression assays were designed separately for each gene using an open-source probe design platform (Kuehn et al., 2022) with standard parameters. HCR probe sequences are provided in Supporting Information: Files S4-S5. Procedures for HCR constitute minor modifications of a recently published protocol (Bruce et al., 2021). For solifuge embryos, we lowered the amount of probe hybridization solution to 148 mL and added in probe stocks at 5× suggested concentration (4.0 μL probe per gene). Confocal imaging was conducted on a Zeiss LSM780 confocal microscope driven by Zen software.

3 | RESULTS

3.1 | Distribution of homeobox duplicates in the embryonic transcriptome of *T. salinarum*

The final assembly of the *T. salinarum* transcriptome yielded a total of 95,457 contigs, a summary length of 86.2 Mb, and an N50 of 1663 bp. Contigs had an average length of 903.8 bp, spanning a range from 11,098 to 194 bp. Inference of completeness using BUSCO v.5.5 and the arthropod-specific BUSCO database estimated the transcriptome to be 88.4% complete with 6% fragmented BUSCOs, and 5.6% missing BUSCOs. The arachnid-specific BUSCO database estimated a similar completeness of 86.6%, with 3.1% fragmented BUSCOs, and 10.3% missing BUSCOs.

Of the 90 homeobox genes queried in the *T. salinarum* transcriptome, 38 (42.22%) could not be identified, leaving 52 loci for further analysis (Figure 3b). The high proportion of missing homeobox genes is attributable to the sampling of only two embryonic stages in the construction of the developmental transcriptome. Of the subset of 52 identified homeobox genes, 14 (26.92%) were deemed uninformative (Figure 3a,c). These genes represented multiple scenarios, including genes duplicated in both *T. salinarum* and representatives of the apulmonate arachnids (*Pax 3/7, Pax4/6, Emx, Barh1*, and *irx*); genes retained in single copy or absent in all surveyed arachnids (*Hbn, Pou6, Isl, Meox, Hhex*, and *Bari*); and genes duplicated only in apulmonate lineages and excluding *T. salinarum* (*Gsc, Nk2.1*, and *Dlx*). Thirty-six (69.23%) of the remaining informative genes were identified as a single copy in *T. salinarum*, while present in duplicate in at least one arachnopulmonate representative (Figure 3a, black arrows; 3c), as exemplified by the well-characterized Hox genes (eight out of 10 present in the *T. salinarum* transcriptome, all as single-copy; Figure 4). Only two (3.85%) genes (*Msx, Lhx2/9*) were identified in which *T. salinarum* shares two or more copies with members of the arachnopulmonates, and members of the apulmonates either retain a single copy, or duplicates in lower number than *T. salinarum* (Figure 3a,c).

For the two homeobox genes wherein *T. salinarum* was observed to have a higher number of duplicates than expected, we validated the evolutionary history of these copies using maximum likelihood analyses to infer gene trees. First, the three duplicates of *Msx* discovered in the *T. salinarum* transcriptome corresponded to one lineage-specific duplication (i.e., with two copies clustering together) and the presence of two *Msx* copies was separately observed in other apulmonate arachnids (e.g., *Ixodes scapularis*; Supporting Information: Figure 1). Second, the two *Lhx2/9* homologs in *T. salinarum* corresponded to homologs of the gene *apterous* in the gene tree of *Lhx* homologs. Whereas other apulmonate arachnids bore a single copy (e.g., *Ixodes scapularis*; *Tetranychus urticae*), arachnopulmonates retain two to

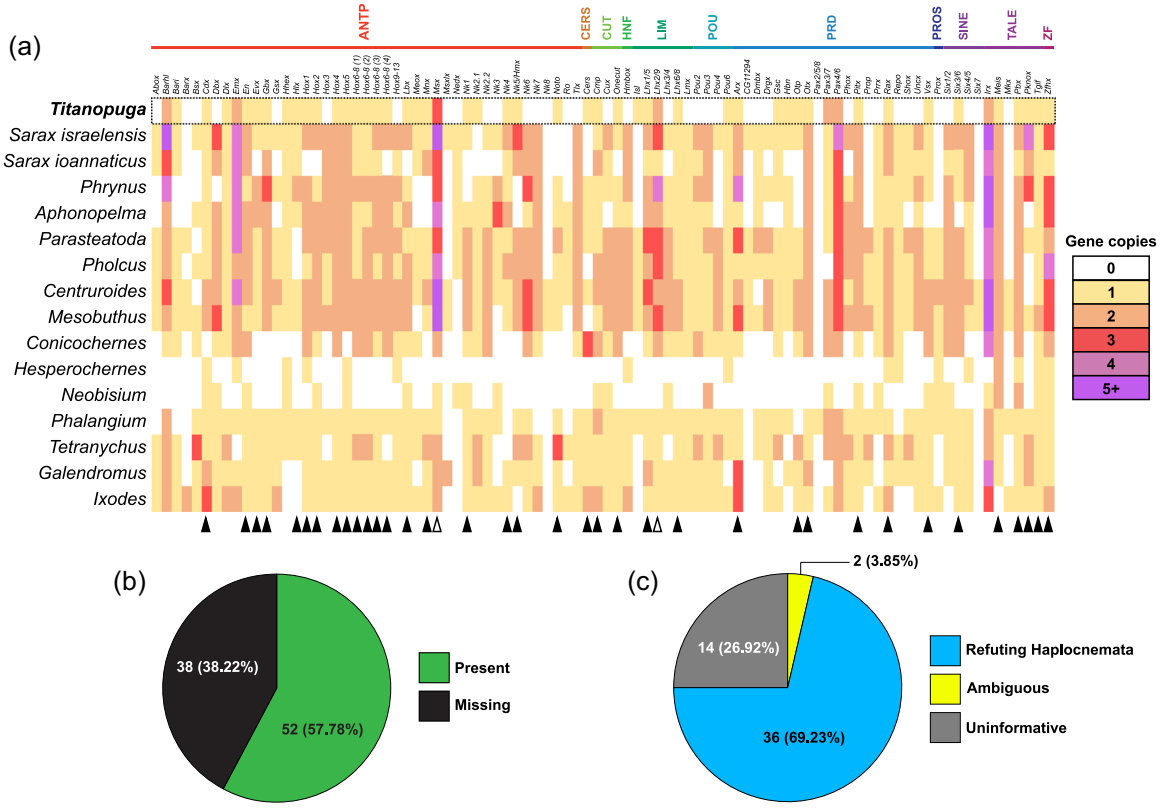

FIGURE 3 (a) Comparison of homeobox repertoires for 16 arachnids refutes the incidence of shared whole genome duplication in *Titanopuga salinarum*. Columns correspond to individual homeobox genes. Colors correspond to numbers of paralogs. Black arrowheads indicate genes for which *T. salinarum* bears a single-copy homolog, whereas duplications occur in at least one arachnopulmonate. White arrowheads indicate two ambiguous cases. (b) Distribution of homeobox genes identified in the embryonic transcriptome of *T. salinarum*. (c) Distribution of homeobox genes present in the embryonic transcriptome of *T. salinarum*, in the context of duplication-based hypothesis testing. [Color figure can be viewed at wileyonlinelibrary.com]

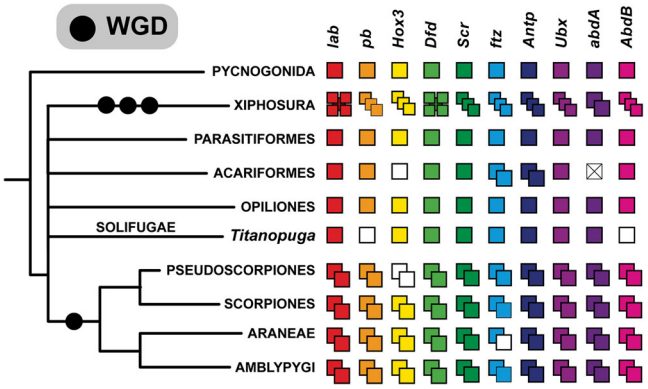

FIGURE 4 Hox gene complement in the solifuge *Titanopuga salinarum* refutes a close relationship to Pseudoscorpiones. Columns and colored squares correspond to each Hox gene. Unfilled squares correspond to absences, not losses. Cross through *abd-A* in *Tetranychus urticae* indicates loss of this Hox gene in the mite genome. Note independent three-fold whole genome duplication events in Xiphosura. Hox surveys in chelicerate orders based on Schwager et al. (2007), Ontano et al. (2021), and Gainett et al. (2021). [Color figure can be viewed at wileyonlinelibrary.com]

four copies. The presence of three clear tetrapulmonate clusters in the *apterous* gene tree suggests that the incidence of two copies in *T. salinarum* does not outright substantiate an arachnopulmonate affinity (Supporting Information: Figure 2).

3.2 | *Expression of* Deformed *and* extradenticle *homologs in* T. salinarum

The anterior Hox gene *Deformed* (*Dfd*) in *T. salinarum* is expressed in the ventral ectoderm and developing limb buds of prosomal segments four and five, corresponding to the developing first (L1) and second (L2) pair of walking legs, although expression appears significantly stronger in the L1 segment (Figure 5a,b). Two stripes of expression are also visible in the posterior boundaries of opisthosomal segments O4 and O5 (Figure 5b). The anterior expression boundary of *Dfd* in the solifuge is comparable to surveys of the single-copy ortholog in Opiliones and Acariformes, and duplicated paralogs in

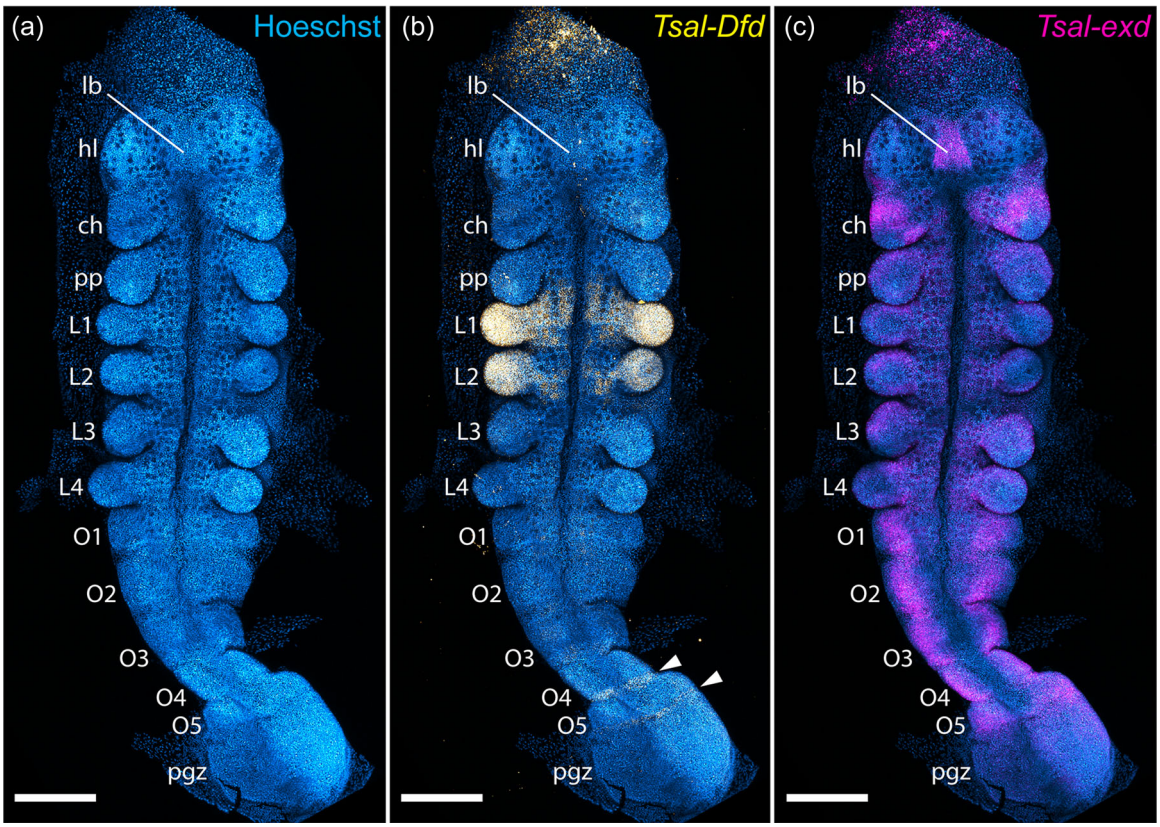

FIGURE 5 Hybridization chain reaction gene expression assay in limb bud stage embryo of *Titanopuga salinarum*. (a) Nuclear counterstain with Hoechst 33342. (b) Expression of *Deformed* is observed in the L1 and L2 (walking leg) segments and as stripes of expression at the posterior boundaries of O4 and O5 (opisthosomal) segments (arrowheads). (c) Expression of *extradenticle* is observed in the labrum; proximal domains extending into the body wall of the prosomal appendages; and in the lateral body wall of opisthosomal segments O1–O5. ch, chelicera; hl, head lobe; lb, labrum; L1, walking leg 1; O1, opisthosomal segment 1; pgz, posterior growth zone; pp, pedipalp. Scale bar: 250 μm. [Color figure can be viewed at wileyonlinelibrary.com]

scorpions, spiders, and horseshoe crabs (Damen et al., 1998; Gainett et al., 2023; Schwager et al., 2017; Sharma et al., 2012b; Telford & Thomas, 1998). However, the posterior prosomal boundary in L2 of *T. salinarum* differs from the L4 boundary observed throughout other surveyed chelicerates, as well as ubiquitous expression of the *Dfd* single copy through the opisthosoma in acariform mites, a domain also observed in the expression of *Dfd-2* in scorpions and *Dfd-B* in horseshoe crabs (Gainett et al., 2023; Telford & Thomas, 1998). Given, however, that RNAi against *Dfd* in the harvestman yielded homeotic transformations of only L1 and L2 toward pedipalpal identity, expression of *Dfd* in L1–L2 of *T. salinarum* is suggestive of the same canonical Hox function in prosomal appendage identity of solifuges.

Expression of the leg gap gene *extradenticle* (*exd*) in *T. salinarum* mirrored the domains observed in harvestmen, acariform mites, scorpions, and spiders (Barnett & Thomas, 2013; Nolan et al., 2020; Pechmann & Prpic, 2009; Sharma et al., 2012a). Strong *exd* expression is observed in the labrum; in all but the distal terminus of the chelicerae; throughout the proximal territory of the developing legs; and in the ventral ectoderm of all prosomal and opisthosomal segments (Figure 5c).

Thus, expression domains across the two selected genes in comparison to other arachnid lineages validated on-target assembly and identification of mRNA sequences in the *T. salinarum* developmental transcriptome.

4 | DISCUSSION

4.1 | The Haplocnemata hypothesis is refuted by a rare genomic change

Given the broad, sweeping impact of WGD on genomic architecture, its signature becomes difficult to mask, effectively providing an immediate influx of hundreds or thousands of potential synapomorphies that can be leveraged to resolve the placement of lineages exhibiting topological instability in traditional morphological or molecular analyses. Such synapomorphies include the shared doubling of microRNA families, temporal and spatial divergence in gene expression, gene tree topologies of ensuing paralogs, and the retention of paralogs of highly conserved developmental genes (Dehal & Boore, 2005; Leite et al., 2018; Schwager et al., 2017; Simakov et al., 2020, 2022). However, the dearth of genomic resources for many chelicerate orders has delayed resolution of this recalcitrant group of arthropods. Here, we applied analyses of homeobox genes to the first developmental transcriptome of a solifuge, a technique previously utilized to resolve the placement of scorpions and pseudoscorpions within Arachnopulmonata (Leite et al., 2018; Ontano et al., 2021). None of the homeobox genes identified in the *T. salinarum* transcriptome supported the inference of a shared WGD in Solifugae. Over 70% of identified homeobox genes are retained as a single copy in *T. salinarum*, while the same loci are retained in duplicate in at least one member of the arachnopulmonates. Only two other loci in *T. salinarum* retained multiple duplicates congruent with the copy numbers observed in the arachnopulmonates. However, gene tree analysis of these loci revealed either a solifuge-specific tandem duplication, or the presence of additional clusters of these paralogs in arachnopulmonates. Successful identification of the homeobox genes (including one Hox gene) was also secondarily validated by fluorescent gene expression assays in a *T. salinarum* embryo. Both the Hox gene *Deformed* and the leg gap gene *extradenticle* had broad congruence in expression domains to single copy orthologs in other apulmonate arachnids, and the duplicate copies present in horseshoe crabs and arachnopulmonates. Thus, despite apparent morphological similarities between Solifugae and Pseudoscorpiones, Haplocnemata is confidently refuted.

This refutation of Haplocnemata precipitates reinterpretation of the eight previously suggested synapomorphies as homoplasies or convergences. However, a post hoc evaluation of the matrix used by Shultz (2007) to recover Haplocnemata would contest their phylogenetic value in the first place (Table 2 and Figure 6). Only three of the eight characters proposed to unite Haplocnemata appear to be unique, unreversed synapomorphies (i.e., found only in Solifugae and Pseudoscorpiones): spiracles present on O3 and O4; elongate, femur-like patellae; and coxae meeting in the ventral midline. The other five characters are either convergent with other taxa, incorrectly scored upon post hoc evaluation, or characters that are not universally applicable across Haplocnemata and were, notably, scored as such by Shultz (2007). As examples, two-segmented chelicerae are common across arachnid orders (Araneae, Amblypygi, Schizomida, Uropygi, and Ricinulei). Shared characteristics of cheliceral articulation found in solifuges and pseudoscorpions are either also present in orders such as Acariformes and Parasitiformes (ventrolateral intrinsic articulation) or absent in basally branching superfamilies of Pseudoscorpiones (dorsolateral carapacal articulation). Notably, Starck et al. (2022) also questioned the homology of the rostrosoma. Comparative anatomical analysis demonstrated that the solifuge rostrosoma is derived exclusively from the epistome, whereas the pseudoscorpion rostrosoma is derived primarily from the labrum. The midventral sternapophysis forming the

TABLE 2 List of putative synapomorphies underlying morphology-based groupings in higher-level chelicerate phylogeny, with explicit character evaluations.

Character	Evaluation
Arachnida	
1. Absence of carapacal doublure	Shared absence
2. Absence of cardiac lobe	Shared absence
3. Absence of pedal gnathobases	Shared absence
4. Absence of movable endites	Shared absence
5. Aerial respiration	Highly homoplastic; not found in all arachnids
6. Anterior or anteroventral mouth orientation	Convergent with Pycnogonida
Haplocnemata	
1. Two-segmented chelicera	Convergent with Araneae, Amblypygi, Schizomida, Uropygi, and Ricinulei
2. Chelicera with ventrolateral intrinsic articulation	Convergent with Acari
3. Chelicera with dorsolateral carapacal articulation	Reversal in basal pseudoscorpion superfamilies
4. Rostrosoma	Absent in Pseudoscorpiones
5. Elongate patellae	Unreversed synapomorphy
6. Midventral sternapophysis	Absent in Pseudoscorpiones; convergent with Palpigradi, Araneae, Uropygi, Amblypygi, Schizomida, Parasitiformes, and Ricinulei
7. Coxae meet at ventral midline	Unreversed synapomorphy
8. Paired spiracles on O3 and O4	Unreversed synapomorphy
Acaromorpha	
1. Pedipalpal coxae broadly fused medially	Convergent with Uropygi and Schizomida
2. Movable subcapitulum	Unreversed synapomorphy
3. Patellotibial joints with hinge articulations	Convergent with Solifugae
4. Tracheae	Convergent with Opiliones, Solifugae, and Pseudoscorpiones
5. Hexapodous larval instar	Unreversed synapomorphy
6. Absence of L3 coxal gland orifices	Convergent with Palpigradi, Uropygi, Schizomida, and Solifugae
Acari	
1. Absence of pygidium	Convergent with Opiliones, Scorpiones, Pseudoscorpiones, Solifugae, and Araneae
2. Aflagellate spermatozoa	Convergent with Solifugae and Palpigradi
3. Stalked spermatophore	Convergent with Pedipalpi, Scorpiones, Pseudoscorpiones, and Solifugae
4. Ovipositor	Convergent with Opiliones
5. Hexapodous larval stage	Convergent with Ricinulei
6. Rutellum	Unreversed synapomorphy
Dromopoda	
1. Femoropatellar joint formed by transverse bicondylar articulation	Autapomorphic state in Solifugae
2. Posterior transpatellar muscle arises from distodorsal surface of femur and patella walls and inserts on anteroventral and posteroventral margins of tibia	Autapomorphic state in Solifugae shared with Schizomida and Ricinulei

(Continues)

TABLE 2 (Continued)

Character	Evaluation
3. Hinge articulation of patellotibial joint	Autapomorphic state in Solifugae shared with Ricinulei and Acari
4. Transverse furrows on prosomal carapace corresponding to margins of segmental tergites	Autapomorphic state in Solifugae shared with Palpigradi and Schizomida
5. Reduced intercoxal sternal region	Unreversed synapomorphy
6. Prosomal endosternite composed of two segmental components	Autapomorphic state in Solifugae
7. Undivided femora on L3 and L4	Reversal in Solifugae; Convergent with Palpigradi, Araneae, Amblypygi, Uropygi, and Schizomida
8. Pretarsal depressor muscle with patellar head	Convergent with Araneae, Amblypygi, Uropygi, and Schizomida
Stomothecata	
1. Stomotheca	Unreversed synapomorphy for crown-group only (absent in stem-group Scorpiones; Dunlop et al., 2008)
2. Coxapophysis of L2	Unreversed synapomorphy
3. Lateral walls of epistome fused to pedipalpal coxae and lumen spanned by transverse muscle	Unreversed synapomorphy
4. Pair of large epistomal arms projecting posteriorly into prosoma; attached to endosternite	Unreversed synapomorphy
5. Pharyngeal dilator muscles attach to epistomal arms	Unreversed synapomorphy
6. Extrinsic muscles of anterior prosomal appendages attach to epistomal arms	Unscored or reversal in Eupnoi; Unscored in Laniatores; no data on Dyspnoi
7. Chelicera with a muscle originating on carapace and inserting on ventral margin of second article	Unscored in a subset of Cyphophthalmi (*Cyphophthalmus*), Eupnoi (*Caddo*), and Laniatores (*Sclerobunus*)
8. Anteriorly placed genital opening	Convergent with Acari

Note: Characters for Arachnida, Haplocnemata, and Stomothecata based on Shultz (2007); characters for Acari, Dromopoda, and Acaromorpha based on Shultz (1990).

ventral component of the beak-like preoral complex is also absent in Pseudoscorpiones, which instead possess a ventral lip of unresolved morphological origin (Starck et al., 2022). Although present in Solifugae, the midventral sternapophysis is convergent with seven other orders (Palpigradi, Araneae, Uropygi, Amblypygi, Schizomida, Parasitiformes, and Ricinulei). The present analysis therefore aligns with the views expressed by Alberti and Peretti (2002), who argued that the long-standing characters supporting Haplocnemata are of debatable value, in light of sperm ultrastructural characters shared by solifuges and acariform mites.

Despite the clear refutation of Haplocnemata by the homeobox gene survey herein, the placement of Solifugae in the chelicerate tree of life nevertheless remains an open question. Yet, a subset of molecular analyses has provided support for the alternative morphological affinity between Solifugae and Acariformes (Poecilophysidea), in turn sister group to Palpigradi (Cephalosomata); included among these are computationally intensive analyses that have implemented site heterogeneous models on matrices carefully curated to remove sites that violate assumptions of the CAT model (Ballesteros et al., 2022; Pepato et al., 2010). The congruence between these analyses and a minority of traditionally overlooked characters (sperm ultrastructure; tagmatization of the prosoma) suggests that a small number of morphological characters better retain phylogenetic signal than a multitude of characters that have been recycled for years across morphological matrices (Shultz, 2007). Indeed, the morphological support previously inferred for Haplocnemata has overshadowed nearly 150 years of noted affinities between solifuges and acariform mites (reviewed by Dunlop, 1999). Originally described by Pickard-Cambridge (1876) and placed in the genus *Poecilophysis*, the rhagidiid mite *Rhagidia kerguelenensis* was suggested as a potential link

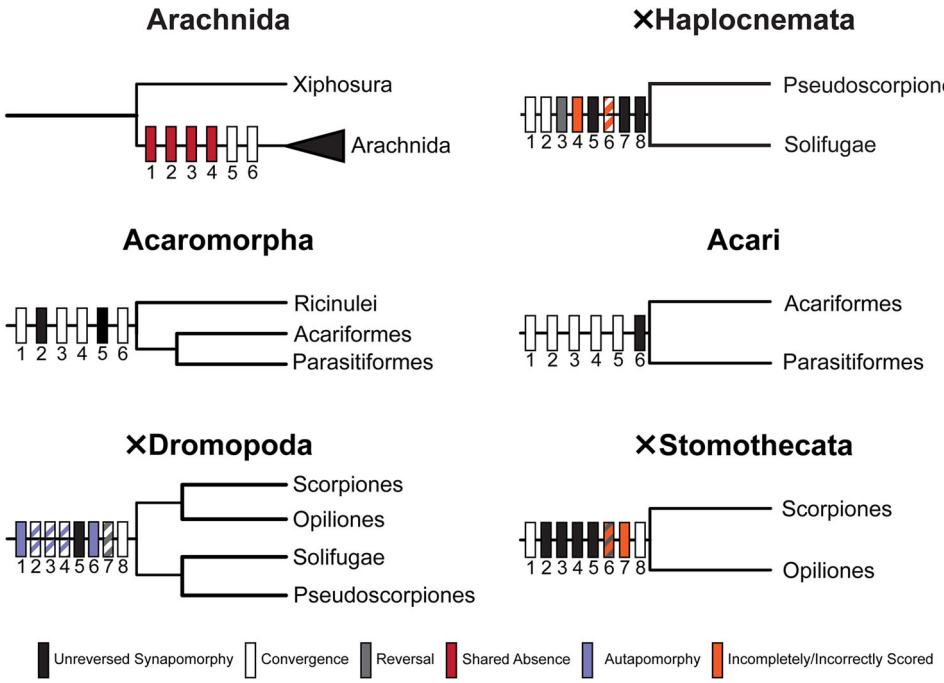

FIGURE 6 Selected morphology-based groupings in higher-level chelicerate phylogeny that reflect high levels of homoplasy and questionable coding. Hypotheses denoted by "X" have been refuted by a rare genomic change (whole genome duplication). [Color figure can be viewed at wileyonlinelibrary.com]

between acariform mites and solifuges on the basis of shared characters of the median eyes, hypertrophic chelicerae, and tagmatic organization, as well as striking similarity in appearance. Banks (1915) and Grandjean (1936, 1954) also acknowledged the similarity in tagmosis and cheliceral architecture of solifuges in comparison to rhagidiids and opilioacarids, respectively. Further support for this relationship was supplied by Alberti (1984), who first recognized the similarity in testis histology between solifuges and acariforms, and characteristics such as cell size, reduction of the nuclear envelope, and acrosome structure of spermatozoa, as potential synapomorphies. Yet, as Dunlop (1999) pointed out, a closer relationship between Acariformes and Solifugae would precipitate the nonmonophyly of Acari (Acariformes + Parasitiformes), a view that challenges broad similarities in morphology, ecology, and postembryonic development of acariforms and parasitiforms. Notably, the monophyly of Acari is now also strongly refuted in phylogenomic analyses, with the addition of a single slowly-evolving lineage (Opilioacariformes, the putative sister group of Parasitiformes) consistently breaking the long-branch attraction between Acariformes and Parasitiformes (Ballesteros, Santibáñez-López, et al., 2019; Ontano et al., 2021).

The recognition that particular morphological character systems may retain high phylogenetic signal substantiates new perspectives on other unresolved phylogenetic relationships among chelicerates. Of note is the presence of a coiled flagellum in the spermatozoa of Ricinulei. This character is shared only by pseudoscorpions and the four tetrapulmonate orders. However, as in the case of Solifugae, an affinity with members of the arachnopulmonates must first be validated by a shared WGD. Thus, we anticipate that the development of additional genomic and transcriptomic resources for the remaining arachnid orders may break the soft polytomy at the base of Euchelicerata.

4.2 | Limitations of morphology in inferences of higher-level chelicerate phylogeny

The observation that eight morphological synapomorphies supporting Haplocnemata instead constitute a series of convergences suggests that a plurality of morphological characters does not equate with phylogenetic confidence or accuracy in higher-level chelicerate phylogeny. Finding eight synapomorphies uniting nodes in higher-level invertebrate phylogeny (i.e., Paleozoic divergences) is rare, and many long-standing relationships among chelicerates are supported by fewer synapomorphies. This refutation of Haplocnemata thus invites careful scrutiny of other interordinal relationships that have been consistently recovered across morphological cladistic analyses in the literature.

One such long-standing morphological hypothesis is Acaromorpha, the clade representing the sister group relationship of Ricinulei and Acari (Acariformes + Parasitiformes). Shultz (1990) suggested a series of six synapomorphies that supported the relationships between these three orders (Table 2). However, of these six characters, only two characters (the presence of a hexapodous larval instar and a movable subcapitulum) are unreversed synapomorphies within the data matrix (Figure 6). Acari themselves also appear poorly supported in the analysis of Shultz (1990), with only a single unreversed synapomorphy (presence of the rutellum), compared to five putatively synapomorphic characters convergent with at least one other arachnid order (Table 2 and Figure 6). The now defunct Dromopoda (Scorpiones + Opiliones + Solifugae + Pseudoscorpiones) is likewise rife with putative synapomorphies that are violated by autapomorphic or convergent traits in Solifugae, as well as broadly convergent characters like the undivided L3 and L4 femora, or the pretarsal depressor muscles with a patellar head (Table 2 and Figure 6). Stomothecata (Scorpiones + Opiliones) as defined in Shultz (2007), is one of the better-supported relationships, with four unreversed synapomorphies (Table 2 and Figure 6). However, unresolved character states in major lineages of Opiliones left two characters that were not validated (i.e., partially scored in the matrix), and the anteriorly placed genital opening observed in Opiliones and Scorpiones is convergent with Acariformes and Parasitiformes. The presence of a stomotheca, likewise, appears convergent within Opiliones and modern Scorpiones (i.e., Orthosterni), given its apparent absence in stem-group scorpion fossils (Dunlop et al., 2008). Despite this suite of morphological similarities substantiating Stomothecata, the absence of a duplicated genome in Opiliones refutes an especially close affinity with Scorpiones, with the latter sharing the arachnopulmonate WGD (Gainett et al., 2021; Leite et al., 2018). Overall, few interordinal relationships proposed by Shultz (1990, 2007) exhibited any nodal support, as measured by bootstrap resampling or Bremer support, likely due to the lability of the characters invoked to support these groupings. The exceptions to this rule were the relationships within Tetrapulmonata, which have been consistently obtained across a variety of data classes and matrices (Bicknell et al., 2019; Giribet, 2002; Regier et al., 2010; Weygoldt & Paulus, 1979; Wheeler & Hayashi, 1998).

The most prominent higher-level grouping advocated by Shultz (1990, 2007) was Arachnida itself, with support for the monophyly of terrestrial chelicerates based on a series of six synapomorphies (Table 2; Shultz, 2007). As previously discussed (Sharma et al., 2021), four of these represented shared absences, characters that are notoriously unreliable in delineating taxa (Figure 6). Of the remaining two putative proposed synapomorphies, an anterior/anterolateral orientation of the mouth is also observed in Pycnogonida (sea spiders), while aerial respiration is (1) plastic within Arachnida, as exemplified by aquatic mites and gilled scorpion fossils (Pepato et al., 2022) and (2) nonuniquely present in other terrestrial animals and therefore prone to homoplasy. Regrettably, Shultz (1990, 2007) never truly tested the monophyly of Arachnida, rooting the tree with Xiphosura and treating the monophyly of Arachnida as *sine qua non* for chelicerate phylogeny. The legacy of those assumptions is found throughout the chelicerate phylogenetic literature, with many of the associated characters recurring in subsequent morphological cladistic analyses and deeply influencing a generation of chelicerate anatomists and paleontologists (Bicknell et al., 2019; Garwood & Dunlop, 2014; Giribet, 2002; reviewed by Giribet, 2018; Sharma et al., 2021). As exemplified by the present study, validation of phylogenetic hypotheses using genomic data sets, in tandem with rigorous and unbiased approaches to phylogenomic inference, is gradually eroding the influence of morphological cladistic approaches to chelicerate phylogeny.

5 | CONCLUSIONS

Whereas a large number of morphological characters does not equate with phylogenetic accuracy in chelicerate phylogeny, the inverse is also true; a paucity of morphological characters does not equate with phylogenetic inaccuracy in Chelicerata. As a prominent example, no single morphological character unites the clade Arachnopulmonata, yet this clade is robustly supported based on a shared WGD (Ontano et al., 2021). Similarly, Panscorpiones (Scorpiones + Pseudoscorpiones) within Arachnopulmonata appears to be united by few morphological characters, such as the shape of the pedipalpal chela and features of mature sperm morphology (e.g., a flagellar tunnel surrounding the axoneme; Alberti, 1995). Thus, phylogenetic hypotheses like Cephalosomata and Poecilophysidea should not be dismissed based on imbalances in putative morphological synapomorphies when compared to groupings like Haplocnemata. For this reason, future investigations of chelicerate must target character systems that exhibit high phylogenetic signal (e.g., sperm ultrastructure; tagmatization) and the developmental mechanisms that underlie their patterning. Discovering the developmental genetic basis for highly plastic characters may also prove to be fertile ground for the study of mechanisms underlying anatomical convergence.

AUTHOR CONTRIBUTIONS

The study was designed by Guilherme Gainett and Prashant P. Sharma. Guilherme Gainett, Catalina Simian, and Hernán Iuri collected specimens and tissues of *T. salinarum*. Guilherme Gainett, Benjamin C. Klementz, and Emily V. W. Setton performed the analyses. Benjamin C. Klementz wrote the first draft, with input from all authors. Alfredo V. Peretti and Prashant P. Sharma funded the work.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available as supplementary material, on NCBI (under embargo), or from the corresponding author.

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