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SYMPOSIUM

The Impact of Whole Genome Duplication on the Evolution of the Arachnids

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Synopsis The proliferation of genomic resources for Chelicerata in the past 10 years has revealed that the evolution of chelicerate genomes is more dynamic than previously thought, with multiple waves of ancient whole genome duplications affecting separate lineages. Such duplication events are fascinating from the perspective of evolutionary history because the burst of new gene copies associated with genome duplications facilitates the acquisition of new gene functions (neofunctionalization), which may in turn lead to morphological novelties and spur net diversification. While neofunctionalization has been invoked in several contexts with respect to the success and diversity of spiders, the overall impact of whole genome duplications on chelicerate evolution and development remains imperfectly understood. The purpose of this review is to examine critically the role of whole genome duplication on the diversification of the extant arachnid orders, as well as assess functional datasets for evidence of subfunctionalization or neofunctionalization in chelicerates. This examination focuses on functional data from two focal model taxa: the spider *Parasteatoda tepidariorum*, which exhibits evidence for an ancient duplication, and the harvestman *Phalangium opilio*, which exhibits an unduplicated genome. I show that there is no evidence that taxa with genome duplications are more successful than taxa with unduplicated genomes. I contend that evidence for sub- or neofunctionalization of duplicated developmental patterning genes in spiders is indirect or fragmentary at present, despite the appeal of this postulate for explaining the success of groups like spiders. Available expression data suggest that the condition of duplicated Hox modules may have played a role in promoting body plan disparity in the posterior tagma of some orders, such as spiders and scorpions, but functional data substantiating this postulate are critically missing. Spatiotemporal dynamics of duplicated transcription factors in spiders may represent cases of developmental system drift, rather than neofunctionalization. Developmental system drift may represent an important, but overlooked, null hypothesis for studies of paralogs in chelicerate developmental biology. To distinguish between subfunctionalization, neofunctionalization, and developmental system drift, concomitant establishment of comparative functional datasets from taxa exhibiting the genome duplication, as well as those that lack the paralogy, is sorely needed.

Introduction

Whole genome duplications are of broad evolutionary interest from the perspective of diversification and body plan disparity. Among the outcomes of systemic gene duplication and the ensuing availability of new genetic material are the segregation of ancestral genes' functions in the daughter copies (subfunctionalization), as well as the acquisition of new functions by daughter copies (neofunctionalization) (Ohno 1970; Lynch and Conery 2000). The latter is of particular interest because it is thought that neofunctionalization could underlie the origins of key molecular and morphological innovations (Bergthorsson et al. 2007; Deng et al. 2010; Anderson et al. 2016; Santibáñez-López et al. 2022). These adaptations may in turn spur cladogenesis, and thus increase net diversification rates, resulting in clades with genome duplications becoming more diverse than their sister groups with unduplicated genomes (Ohno

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1970; Wagner et al. 2003; Dehal and Boore 2005; Vamosi and Dickinson 2006; Walden et al. 2020). However, the precise relationship between genome duplications and speciation rate is complex, as highlighted by many angiosperm examples. Specifically, the timing of the duplication event is thought to be an additional factor that influences its overall impact on net diversification rate (Wood et al. 2009; Mayrose et al. 2011; Landis et al. 2018).

Other models postulate that the incidence of duplicated copies may drive the same outcome of increased net diversification, but by different means; genome duplications may reduce risks of lineage extinction through diverse mechanisms, such as functional redundancy of gene copies, robustness to deleterious mutations, and increased potential for adaptation (Crow and Wagner 2006). These models are not mutually exclusive and both processes may play roles in different contexts across taxa with duplicated genomes, especially given the prolonged lifespans of gene copies (even those bound for eventual gene loss) in groups with large effective population sizes (Johri et al. 2022). For these reasons, the retention of duplicate copies, the spatiotemporal expression dynamics of the paralogs, the identification of neofunctionalized copies, and the diversification dynamics of the clades that harbor them, are all of interest from the perspective of evolutionary developmental biology and comparative genomics (Wagner et al. 2003; Crow and Wagner 2006; Crow et al. 2006; Simakov et al. 2020, 2022).

Chelicerates (e.g., spiders, scorpions, mites, and horseshoe crabs) are a diverse subdivision of Arthropoda that span over 120,000 described species to date, and are among the handful of metazoan lineages that exhibit evidence for multiple ancient whole genome duplications (Fig. 1A). This subphylum of invertebrates is notable for its recalcitrant phylogenetic relationships, as well as numerous morphological, ecological, and molecular innovations within the group, such as webs, silks, venoms, extreme miniaturization, and eyes of diverse arrangements and capabilities (Dunlop 2018; Garb et al. 2018; Giribet 2018). The fossil record of chelicerates suggests that the group was a well-established component of early Paleozoic ecosystems, with nearly all extant orders present by the Devonian (Dunlop 2010). Today, chelicerates are found in all major terrestrial and aquatic habitats, and exhibit a complex history of terrestrialization, paralleling the case of Mandibulata (the remaining Arthropoda) (Sharma 2017).

The traditional understanding of chelicerate phylogeny was that aquatic orders like Pycnogonida (sea spiders), Xiphosura (horseshoe crabs), and Eurypterida (the extinct sea scorpions) formed a grade with respect to a monophyletic Arachnida—implying a single colonization of land in the common ancestor of the arachnids (Weygoldt and Paulus 1979; Shultz 1990, 2007; Regier et al. 2010). The minority view, that arachnids may not constitute a single monophyletic group (Hammen 1977; Dunlop 1998), has been repeatedly encountered and surprisingly well-supported by phylogenomic analyses (Pepato et al. 2010; Sharma et al. 2014a; Ballesteros and Sharma 2019). While initially thought to be an artifact, the recovery of Xiphosura (as well as other fossil aquatic orders like sea scorpions) has been robustly defended by new datasets and analyses that emphasize dense taxonomic sampling of extant chelicerate orders; sophisticated approaches to modeling evolutionary processes; and inclusion of morphological data, both as standalone analyses and combined with molecular datasets (Ballesteros and Sharma 2019; Ballesteros et al. 2019, 2022; Ban et al. 2022; reviewed by Sharma et al. 2021a). It is likely that there were at least two colonizations of land near the base of the chelicerate tree of life, together with secondary colonizations of aquatic habitats in one group of extinct gilled scorpions from the Devonian and multiple extant lineages of aquatic mites (Dunlop 2010; Pepato et al. 2018, 2022) (Fig. 1B). One lineage of these aquatic mites may even have tertiarily recolonized terrestrial habitats (Pepato et al. 2022)—close parallels of the evolutionary history of Mandibulata (myriapods, hexapods, and crustaceans) on the other side of the arthropod tree of life.

A fascinating facet of chelicerate evolution lies in the dynamism of their genomes. The recent discovery of waves of genome duplication within Chelicerata added a new dimension to their biological complexity. Two branches in the chelicerate tree of life exhibit genome duplications. The first unites Arachnopulmonata, a group of six orders that ancestrally possessed book lungs (secondarily lost in pseudoscorpions and miniatured spiders) (Sharma et al. 2014a; Schwager et al. 2017). This genome duplication was originally discovered through surveys of Hox gene patterns in embryonic spiders; retrieval of Hox sequences using degenerate PCR yielded two copies of four separate Hox genes, which differed both in sequence and in the specifics of their expression patterns (Damen et al. 1998; Abzhanov et al. 1999; Schwager et al. 2007). Subsequently, transcriptome- and genome-based surveys showed that nearly all Hox genes were duplicated in both spiders and scorpions, with gene tree topologies supporting a shared origin of the duplication in these two arachnid orders (Sharma et al. 2014a; Schwager et al. 2017). This result has been corroborated by subsequent genome sequencing efforts, which now span nearly 40 arachnid species across six orders (Table 1) (reviewed by Garb et al. 2018 and Gainett

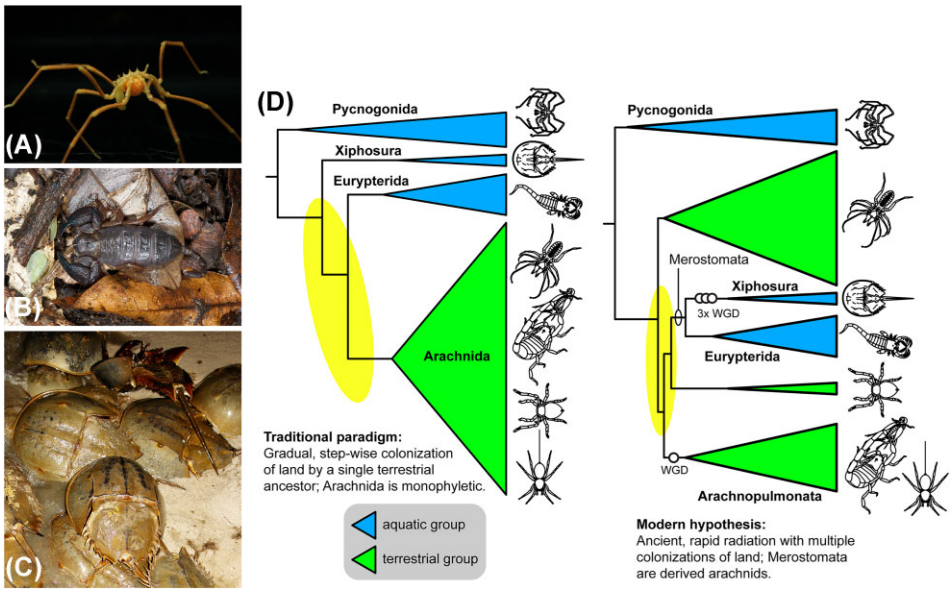

Fig. 1 Exemplars of chelicerate diversity and changing views of phylogeny. (A) Male of the sea spider *Ammothea glacialis* carrying eggs (photo: T. Higham). (B) The hormurid scorpion *Opisthacanthus* cf. *asper* (photo: G. Giribet). (C) The horseshoe crab *Limulus polyphemus* (photo: P. Funch). (D) Traditional and emerging views of chelicerate phylogeny. Left: topology based on cladistic morphological phylogeny of Shultz (2007) with 59 taxa and 202 morphological characters. Right: topology based on total evidence maximum likelihood analysis of Ballesteros et al. (2022) with 514 taxa, 259 morphological characters, and 152 loci

et al. 2021; Ontano et al. 2021; Kuntner 2022; Miller et al. 2023).

The remaining three genome duplications are concentrated on the branch subtending the modern horseshoe crabs and are thought to be relatively recent (Kenny et al. 2016; Shingate et al. 2020; Nong et al. 2021). As with spiders, evidence for the duplicated condition of the horseshoe crab genome began with degenerate PCR assays, which recovered up to four copies per Hox gene in an early work (Cartwright et al. 1993). The architecture of horseshoe crab Hox clusters is now far better understood due to chromosomal-level genome assemblies for two of the three living genera (Shingate et al. 2020; Nong et al. 2021).

Ancient whole genome duplications are comparatively rare in metazoans, by contrast to the botanical or mycological literature. A handful of cases is known, with the best characterized of these being the waves of genome duplication at the base of the vertebrates (Dehal and Boore 2005; Putnam et al. 2008; Simakov et al. 2020). Yet genome duplications are compelling phenomena that serve as catalysts for new avenues of inquiry and hypothesis-testing. With respect to phylogenetics, genome duplications are often useful arbiters for breaking polytomies, in that they serve as rare genomic changes—complex characters that exhibit low levels of homoplasy (Salichos and Rokas 2014; One Thousand Plant Transcriptomes Initiative 2019). Apropos, the discovery of the arachnopulmonate duplication lent itself as a rare genomic change to test the placement of Pseudoscorpiones, an unstable group of rapidly evolving and small-bodied arachnids (Ballesteros et al. 2022; Ontano et al. 2022). The first developmental and genomic resources for pseudoscorpions revealed shared patterns of duplication with the remaining arachnopulmonates and placed them as the likely sister group of scorpions (Ontano et al. 2021), with downstream implications for the evolutionary origin of arachnopulmonate venoms (Santibáñez-López et al. 2018; Krämer et al. 2019). Relationships of other parts of the chelicerate tree of life remain obscured by conflicting signal surrounding an ancient rapid radiation of apulmonate arachnid orders, but these are being sequentially illuminated by dedicated improvements in sampling of enigmatic and poorly studied taxa (Ballesteros et al. 2021; Ban et al. 2022; Ontano et al. 2022; Pepato et al. 2022).

The coincidence of these macroevolutionary processes in a clade with multiple genome duplications makes Chelicerata a useful point of comparison for testing many of the hypotheses pertaining to the effect of genome duplication on evolutionary history. In particular, the incidence, retention, and divergence of paralogs of embryonic patterning genes has been explicitly invoked as an explanatory vehicle for the evolutionary success (i.e., species richness) and morphological diversification in spiders (Schomburg et al. 2015; Turetzek et al. 2015, 2017; Harper et al. 2021). Specific contexts wherein spiders' evolutionary success or mor-

Table 1 Available genomes for Chelicerata. Note that the *Nymphon striatum* (sea spider) and *Acanthoscurria geniculata* (tarantula) genomes exhibit assembly anomalies and are highly fragmented

Order	Species	Reference	Genome size (GB)
Acariformes	*Dinothrombium tinctorium*	Dong et al. (2018)	0.18
Acariformes	*Leptotrombidium deliense*	Dong et al. (2018)	0.12
Acariformes	*Tetranychus urticae*	Grbić´ et al. (2011)	0.09
Araneae	*Acanthoscurria geniculata*	Sanggaard et al. (2014)	>6
Araneae	*Anelosimus studiosus*	Purcell and Pruitt (2019)	2.22
Araneae	*Araneus ventricosus*	Kono et al. (2019)	3.66
Araneae	*Argiope bruennichi*	Sheffer et al. (2021)	1.67
Araneae	*Caerostris darwini*	Kono et al. (2021)	1.58
Araneae	*Caerostris darwini*	Babb et al. (2022)	1.81
Araneae	*Caerostris extrusa*	Kono et al. (2021)	1.42
Araneae	*Dysdera silvatica*	Sa´nchez-Herrero et al. (2019)	1.7
Araneae	*Hylyphantes graminicola*	Zhu et al. (2022)	0.93
Araneae	*Latrodectus hesperus*	Thomas et al. (2020)	1.14
Araneae	*Loxosceles reclusa*	Thomas et al. (2020)	3.26
Araneae	*Parasteatoda tepidariorum*	Schwager et al. (2017)	1.45
Araneae	*Pardosa pseudoannulata*	Yu et al. (2019)	4.26
Araneae	*Stegodyphus dumicola*	Liu et al. (2019)	4.29
Araneae	*Stegodyphus mimosarum*	Sanggaard et al. (2014)	2.74
Araneae	*Tetragnatha kauaiensis*	Cerca et al. (2021)	1.08
Araneae	*Trichonephila antipodiana*	Fan et al. (2021)	2.29
Araneae	*Trichonephila clavipes*	Babb et al. (2017)	2.44
Araneae	*Uloborus diversus*	Miller et al. (2023)	1.98
Opiliones	*Phalangium opilio*	Gainett et al. (2021)	0.58
Parasitiformes	*Dermacentor silvarum*	Jia et al. (2020)	2.76
Parasitiformes	*Galendromma occidentalis*	Hoy et al. (2016)	0.15
Parasitiformes	*Haemaphysalis longicornis*	Jia et al. (2020)	2.59
Parasitiformes	*Hyalomma asiaticum*	Jia et al. (2020)	1.78
Parasitiformes	*Ixodes persulcatus*	Jia et al. (2020)	2.04
Parasitiformes	*Ixodes scapularis*	Jia et al. (2020)	1.77
Parasitiformes	*Neoseiulus cucumeris*	Zhang et al. (2019)	0.17
Parasitiformes	*Rhipicephalus microplus*	Jia et al. (2020)	2.56
Parasitiformes	*Rhipicephalus sanguineus*	Jia et al. (2020)	2.12
Parasitiformes	*Varroa destructor*	Cornman et al. (2010)	0.57
Pseudoscorpiones	*Cordylochernes scorpioides*	Ontano et al. (2021)	2.81
Pycnogonida	*Nymphon striatum*	Jeong et al. (2020)	0.74
Scorpiones	*Centruroides sculpturatus*	Schwager et al. (2017)	0.93
Scorpiones	*Mesobuthus martensii*	Cao et al. (2013)	1.13
Xiphosura	*Carcinoscorpius rotundicauda*	Shingate et al. (2020)	1.67
Xiphosura	*Carcinoscorpius rotundicauda*	Nong et al. (2021)	1.7
Xiphosura	*Limulus polyphemus*	Kenny et al. (2016)	1.8
Xiphosura	*Tachypleus tridentatus*	Nong et al. (2021)	1.7

phological diversity has been linked to genome duplication include justifications for new spider genomes (Fan et al. 2021; Miller et al. 2023), interpretations of single-cell RNA-seq datasets in spider embryogenesis (Leite et al. 2022), explanations for the diversity of spider appendages (Turetzek et al. 2017), and descriptions of the

morphogenesis of spider eyes (Janeschik et al. 2022). The broader influence of whole genome duplication on chelicerate evolutionary history remains poorly understood.

The purpose of this review is to examine critically the evidence for a correlation between incidence of genome duplication and species richness, as well as scrutinize available evidence for sub- and neofunctionalization across Chelicerata. I subsequently highlight the significance of developmental system drift as a valuable alternative explanation for some of the spatiotemporal expression dynamics that have been described for ancient arachnopulmonate gene copies resulting from whole genome duplication.

Does whole genome duplication correlate with greater species richness in Chelicerata?

Within Metazoa, vertebrates are the archetype for the influence of whole genome duplication on evolutionary history. There is a positive correlation between the number of genome duplications and the species richness of the ensuing clades, with vertebrates (two-fold whole genome duplication) exhibiting greater species richness than their sister taxon by an order of magnitude. Within vertebrates, Teleostei (additional, lineage-specific whole genome duplication) even more dramatically outnumber their sister group, the Holostei (bowfins and gars) (Crow et al. 2006). Within the teleost fishes, Salmoniformes are again an order of magnitude more diverse than their sister group, the Esociformes (Christensen and Davidson 2017). Across Metazoa, this relation holds for diverse invertebrate groups spanning a range of divergence times, such as coleoid cephalopods, the coral genus *Acropora*, and two large clades within Gastropoda (Hallinan and Lindberg 2011; Mao and Satoh 2019).

Within Arachnopulmonata, most of the described diversity lies within Araneae (spiders) alone (ca. 50,000 described species). The remaining arachnopulmonates are mesodiverse (described species in the thousands; scorpions and pseudoscorpions) or microdiverse orders (described species in the low hundreds; Uropygi, Schizomida, and Amblypygi). The diversity of spiders is rivaled by Acariformes, a diverse group of mites that includes numerous lineages of parasites and highly miniaturized taxa, and whose described diversity is almost certainly a poorly understood fraction of its extant biodiversity. The sister group of Acariformes is similarly not evident, with the monophyly of Acari (Acariformes + Parasitiformes) presently in question (Pepato et al. 2010; Ballesteros et al. 2022; Ban et al. 2022; Ontano et al. 2022). However, if Acari were monophyletic, the combined diversity of Acariformes and Parasitiformes would easily surpass that of spiders, both with regard to described number of species, as well as ecological and morphological diversity. In addition, mesodiverse and microdiverse orders are similarly present outside of Arachnopulmonata, obscuring a clear trend of increased diversification as a consequence of the arachnopulmonate duplication (Fig. 2).

The marked exception to the trend of genome duplication and species richness is Xiphosura. Three rounds of genome duplication are understood to have occurred along the branch subtending modern horseshoe crabs, yet this group is characterized by low net diversification rates throughout its considerable fossil record, a depauperate modern fauna, and external morphological stasis (Dunlop 2010; Obst et al. 2012; Bicknell et al. 2019; Bicknell and Pates 2020). The four extant species of Xiphosura exhibit similar genomes and levels of paralogy, as inferred from multiple genome sequencing projects, but the exact timing of the genome duplications is difficult to infer, due to the absence of closely related, unduplicated taxa (Kenny et al. 2016; Shingate et al. 2020; Nong et al. 2021). Paleontologists have conjectured that all three modern horseshoe crab genera must exceed the early Mesozoic in age, based on a putative *Tachypleus* fossil from the Triassic (*T. gadeai*), whose interpretation has in turn influenced non-parametric extrapolations of xiphosuran clade ages (Lamsdell and McKenzie 2015). However, based upon inter-paralog distances and distributions of K_s (distributions of synonymous substitutions per site among paralogous genes), it is thought that at least one of the three duplications subtending the extant Xiphosura must be relatively recent (Roelofs et al. 2020), an inference consistent with divergence time estimates of the three extant genera in the early Cretaceous (Obst et al. 2012), as well as the decades-old observation that all three Pacific horseshoe crab species (in the genera *Carcinoscorpius* and *Tachypleus*) are capable of hybridizing to produce viable offspring (Sekiguchi and Sugita 1980). If *Tachypleus* were indeed Triassic in age, this would make the three Pacific Xiphosura the oldest known case of hybridizing species, exceeding the sturgeon–paddlefish hybrids in age (Káldy et al. 2020), in addition to having among the most slowly evolving metazoan genomes documented to date. A more plausible interpretation is that the Triassic *Tachypleus* represents a case of overconfidence in ascribing fossils to crown group lineages, exacerbated by the external morphological stasis exhibited by many fossil Xiphosura. Assumptions about the placement

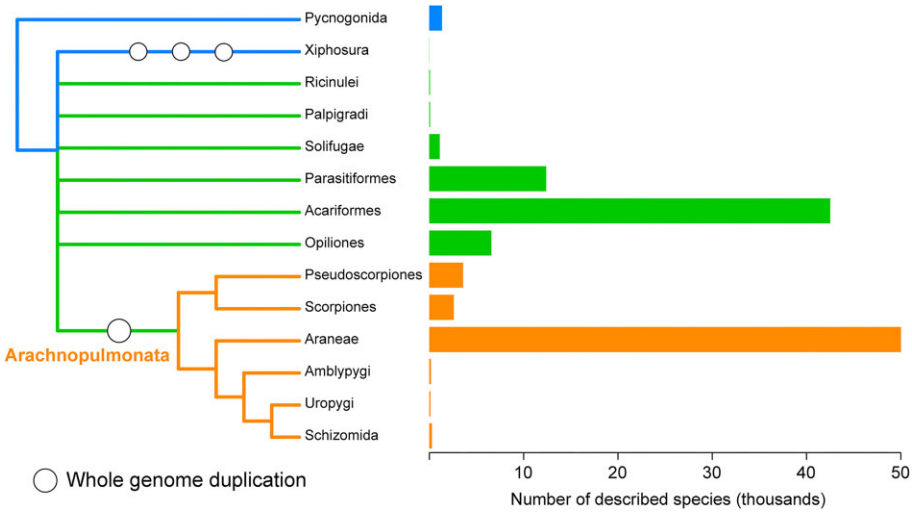

Fig. 2 Described species richness of extant chelicerate orders. Circles on nodes correspond to whole genome duplication events. Left-hand panel: cladogram of extant chelicerate orders (branch lengths not to scale) based on Ballesteros et al. (2022), with unstable nodes collapsed. Colors correspond to exclusively aquatic orders (blue), apulmonate arachnid orders (green), and Arachnopulmonata (orange)

of *T. gadeai* and its influence on molecular divergence time estimates could be assessed via statistically rigorous parametric tests of tip-dated molecular phylogenies, with and without the inclusion of this fossil.

The disputes over clade ages notwithstanding, even the youngest proposed date of crown Xiphosura (Obst et al. 2012) does not help explain their deviation from the correlation between genome duplication and species richness. Many of the plant and animal lineages that exhibit heightened net diversification rates after genome duplications are of comparable, or younger, age to crown Xiphosura, disfavoring the interpretation that horseshoe crabs have not had sufficient time to diversify in the wake of genome duplication (Hallinan and Lindberg 2011; Christensen and Davidson 2017; Landis et al. 2018; Mao and Satoh 2019).

As a simple test of the effect of whole genome duplication on species richness, I compared values of species richness for chelicerate orders with genome duplications (arachnopulmonates and xiphosurans) to those without duplications (apulmonate arachnids and sea spiders), using as a proxy the number of described species per order (Sharma 2023). There is statistically no difference in the means of described species between these two groups ($t = 0.114$; $P = 0.91$), even upon removing the exclusively aquatic groups and retaining only arachnid orders ($t = 0.094$; $P = 0.93$). Thus, the distribution of species richness across Chelicerata does not obviously substantiate the hypothesis that incidence of genome duplications is correlated with increases in net diversification rate.

How strong is the evidence for neofunctionalization as a driver of chelicerate evolution?

If the relationship between incidence of genome duplication and species richness is not readily evident in Chelicerata, another avenue for investigation is the impact of systemic paralogy (i.e., widespread incidence of duplicated genes) on chelicerate body plan disparity. Specifically, the spatiotemporal expression dynamics of paralogous genes that have resulted from whole genome duplication are of value for gauging the impact of new genes on morphological innovations. By comparison to vertebrate models, chelicerate models are few in number of study systems and in number of comparative developmental datasets per study system, particularly from the perspective of functional data. Most of the seminal works on comparative gene expression and gene function in Chelicerata have focused on spiders (Damen et al. 1998; Akiyama-Oda and Oda 2003; Schwager et al. 2015). Indeed, the leading model system for study of chelicerate developmental biology is the house spider *Parasteatoda tepidariorum*, which features access to advanced genomic resources, maternal and embryonic gene silencing, and optimized techniques for single-blastomere injections (Mcgregor et al. 2008; Hilbrant et al. 2012; Posnien et al. 2014; Schomburg et al. 2015; Schönauer et al. 2016; Pechmann et al. 2017; Bednarek et al. 2019; Akiyama-Oda and Oda 2020). The discovery of widespread retention of ancient paralogs in the *P. tepidariorum* genome offered a promising avenue for interrogating the pro-

cesses of sub- and neofunctionalization in a tractable model system, as well as for potentially discovering the developmental genetic mechanisms that precipitated the success of spiders (Leite et al. 2016, 2018; Schwager et al. 2017). Most recently, the advent of the first single-cell and single-nucleus RNA sequencing has facilitated precise characterization of spatiotemporal dynamics of gene copies (Akiyama-Oda et al. 2022; Leite et al. 2022).

Among the most intensively studied duplicated genes in spiders are the Hox genes, due to their central role in body plan patterning across Bilateria, as well as the availability of benchmarked functional datasets from insect and non-arachnopulmonate chelicerate models as points for comparison (Telford and Thomas 1998; Jager et al. 2006; Schwager et al. 2017). Based upon syntenic patterns across arachnopulmonate genomes, including recent chromosomal-level datasets, it is understood that arachnopulmonates ancestrally bore two Hox clusters that resulted from their shared whole genome duplication event (Schwager et al. 2017; Fan et al. 2021; Miller et al. 2023). Evidence for the subfunctionalization of Hox gene copies is most strongly substantiated by the dynamics of the posterior Hox genes. In both scorpions and spiders, copies of *Antennapedia*, *Ultrabithorax*, *abdominalA*, and *AbdominalB* exhibit spatially distinct anterior expression boundaries, which coincide with morphological changes in segment identity (typically with respect to the paired appendage borne by a given segment) (Sharma et al. 2014a; Schwager et al. 2017). For example, in scorpion embryos, the anterior boundary of *abdominalA-1* corresponds to the pectine-bearing segment, whereas the anterior boundary of its paralog, *abdominalA-2*, corresponds to the first book lung-bearing segment (Sharma et al. 2014a). In spiders, the anterior boundaries of the same pair of paralogs correspond to the segments bearing the posterior respiratory organ pair (the tracheal tubules) and the anterior spinneret, respectively (Schwager et al. 2017). These patterns substantiate an evolutionary scenario wherein the availability of new Hox genes facilitated subdivision of expression domains and thus greater heteronomy of posterior body segments in Arachnopulmonata (Sharma et al. 2014b) (Fig. 3).

However, functional data supporting divergent roles of spider Hox paralog pairs are non-existent. The only available data addressing Hox gene function in *P. tepidariorum* have targeted individual copies (not both pairs) with maternal RNAi (Khadjeh et al. 2012; Pechmann et al. 2015). In one case where a duplicate copy of an anterior Hox gene was targeted (*labial-2*), no observable phenotype was recorded and the onset of expression was observed to be later than the copy that yielded a phenotype (Pechmann et al. 2015). More generally, the prosoma (anterior tagma) of spiders is not markedly different from that of non-arachnopulmonate arachnids, and thus differences in expression domains of duplicated pairs of Hox genes do not correlate clearly with morphological differences between arachnid orders at the level of body plan evolution (Gainett et al. 2023). The opisthosoma (posterior tagma), which exhibits greater evolutionary lability and a broader range of adaptations within arachnopulmonates (e.g., the scorpion "tail;" the spider spinneret) compared to other arachnid orders, seems like a more promising target for understanding the roles of duplicated Hox genes, especially given the correlation between anterior expression boundaries and segmental identities described above. However, expression levels of posterior Hox genes are difficult to disrupt using RNAi, due to limited efficiency of available approaches against these candidates (Khadjeh et al. 2012; E.V.W. Setton, *personal communication*; E.E. Schwager, *personal communication*). Many of the Hox duplicates exhibit no phenotypic effects when targeted individually, whereas the technique of double-knockdown is known to have low penetrance. In addition, the posterior Hox genes likely exhibit posterior prevalence, meaning that more than two genes' paralogs may need to be disrupted simultaneously to interrogate the patterning of organs like tracheal tubules and spinnerets (Khadjeh et al. 2012).

A separate complication for detecting neofunctionalization using gene expression alone is the observation that anterior Hox genes exhibit labile posterior boundaries across arachnid orders, but these posterior expression boundaries do not correspond strongly to shifts in identity between adjacent segments. As an example, both *Deformed* paralogs of spiders and the single-copy *Deformed* of a harvestman are restricted to the L1–L4 (walking leg) segments, whereas the *Deformed-2* copy of a scorpion and the single-copy *Deformed* homolog of a mite are both expressed from the L1 segment to the posterior terminus (Telford and Thomas 1998; Schwager et al. 2017; Gainett et al. 2023). Available RNAi datasets in arachnids have shown that anterior Hox boundaries are more significant than posterior boundaries in predicting phenotypic spectra, which consistent with the idea that not all expression domains of transcription factors may be functional (Li et al. 2008; Fisher et al. 2012). For this reason, expression data alone may not be sufficient to infer sub-or neofunctionalization, despite advancements in resolution of gene expression (Leite et al. 2022).

Other investigations of *P. tepidariorum* paralogs have explored the dynamics of duplicated proximo-distal axis-patterning genes. One of the best known cases of putative neofunctionalization in chelicerates is the as-

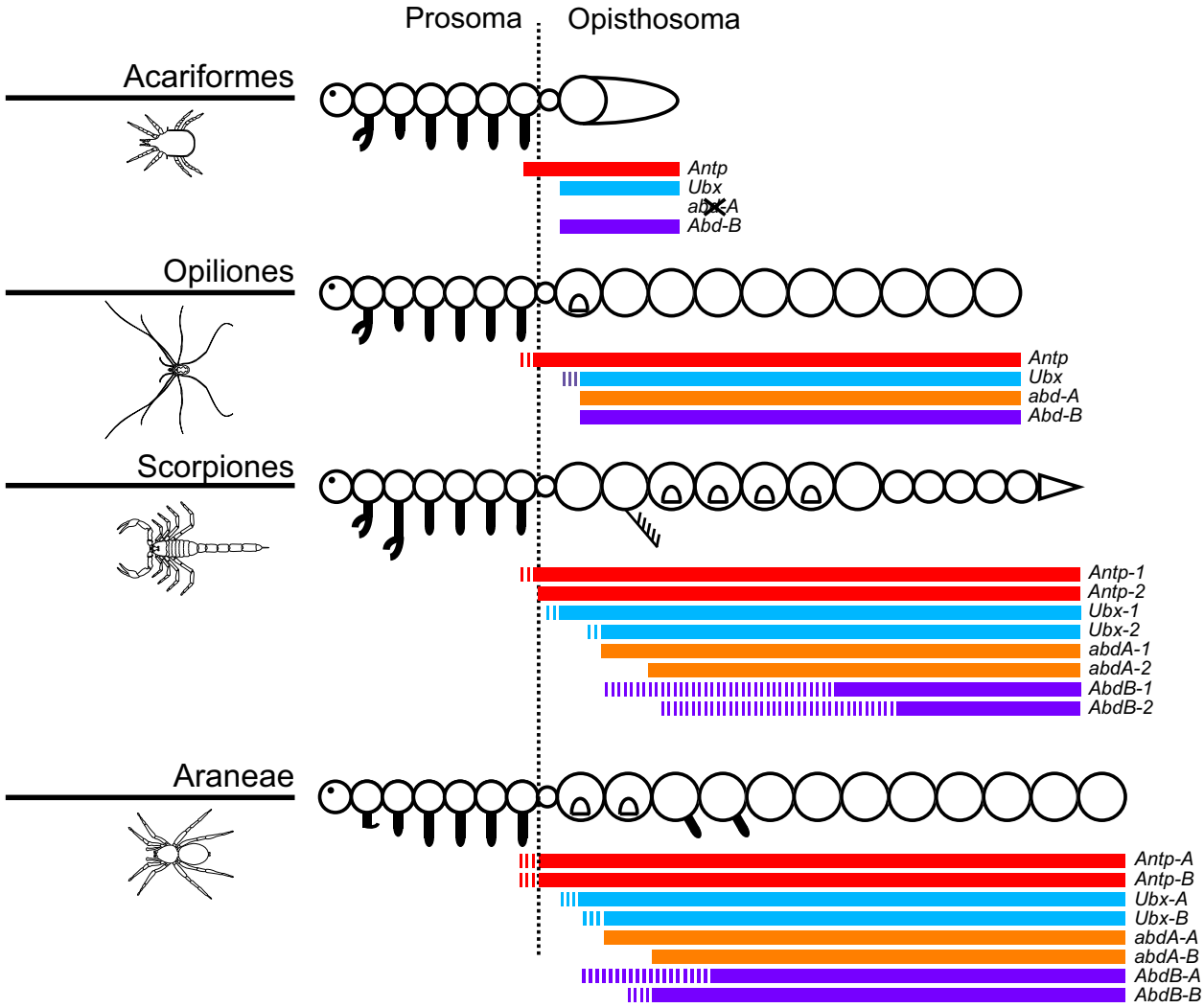

Fig. 3 Expression domains of posterior Hox genes and their paralogs in the mite *Archegozetes longisetosus*, the harvestman *Phalangium opilio*, the scorpion *Centruroides sculpturatus*, and the spider *P. tepidariorum*. Circles correspond to segments. Icons correspond to types of paired organs, such as chelicerae, pedipalps, book lungs, pectines, and spinnerets, following the convention of Sharma et al. (2014b). Solid bars correspond to strong expression domains throughout the indicated segments; dashed bars correspond to weaker expression domains restricted to parts of the indicated segments

sociation between *dachshund-2* and the origin of the patella, the fourth podomere of the spider leg. Across Arthropoda, *dachshund* is canonically expressed in medial segments of the developing walking leg and its abrogation is associated with the deletion of those medial podomeres (Dong et al. 2001; Angelini and Kaufman 2004; Angelini et al. 2012). In spiders, one copy, *dachshund-1*, is expressed as a single medial domain, whereas its paralog, *dachshund-2*, exhibits a proximal domain as well as a distal ring domain that localizes to the patella–tibia boundary (Turetzek et al. 2015). RNAi against *dachshund-2* resulted in the loss of the distal patella boundary and a fusion of the patella with the tibia; RNAi against *dachshund-1* was not attempted or the results were not reported in that work. The authors concluded that neofunctionalization of *dachshund-2* to acquire a novel role in podomere boundary formation precipitated the origin of the arachnid patella (Turetzek et al. 2015). Thus, a new gene gave rise to a morphologically novel trait.

While this scenario is compelling for its mechanistic elegance, it is inconsistent both with the evolutionary history of the gene and with the evolutionary history of the taxon (Fig. 4). The origin of *dachshund-2* is attributable to the whole genome duplication subtending Arachnopulmonata; gene trees of *dachshund* homologs unambiguously support an origin of *dachshund-2* in the common ancestor of the arachnopulmonates (Nolan et al. 2020; Ontano et al. 2021). However, the patella unambiguously evolved

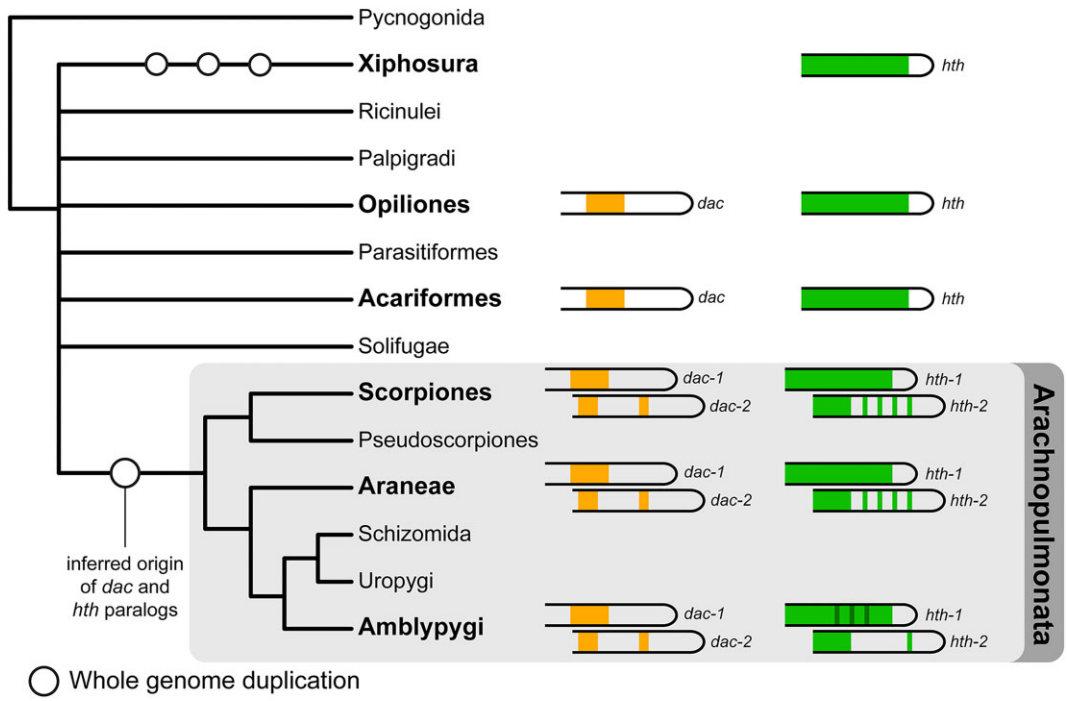

Fig. 4 Schema of expression dynamics of *dachshund* and *homothorax* homologs in Chelicerata. Note that *dachshund* paralogs are restricted to Arachnopulmonata, and thus postdate the origin of the patella, which is present in apulmonate arachnid orders like Opiliones and Acariformes. Similarly, *homothorax* paralogs are also restricted to Arachnopulmonata and do not reflect a spider-specific duplication; note that only one *homothorax* paralog of the horseshoe crab *L. polyphemus* has been surveyed for embryonic gene expression (Sharma et al. 2015)

prior to the emergence of the arachnopulmonates, being present in non-arachnopulmonate arachnid orders with unduplicated genomes (e.g., Opiliones, Parasitiformes, and Acariformes), Xiphosura, and possibly Pycnogonida as well (Snodgrass 1952; Shultz 1989). Thus, groups with single-copy homologs of *dachshund* bear a patella, whereas Xiphosura have patellae and many *dachshund* paralogs that are not orthologs of spiders' *dachshund-2*. As the phylogenetic age of the trait predates the phylogenetic age of its putative causal gene, we can rule out a causal relationship between the origin of *dachshund-2* and the origin of the patellar segment. These patterns of trait and gene copy distribution bespeak the presence of another, as yet unidentified causal gene that underlies the origin of the patella.

Similar interpretations of evolutionary significance were placed on the paralogs of *homothorax*, which exhibits distinct expression patterns across spiders (Turetzek et al. 2017). In proximo-distal axis patterning of mandibulates, *homothorax* plays a role in the establishment of proximal leg segments, together with *extradenticle*; *homothorax* plays an additional and separate role as in establishing antennal fate together with *Distal-less* in the fruit fly, *Drosophila melanogaster* (Dong et al. 2001; Dong et al. 2002; Angelini and Kaufman 2004; Ronco et al. 2008). In spiders, one copy (*homothorax-1*) is typically expressed as a continuous domain from the body wall to the metatarsal boundary. The other copy, *homothorax-2*, is expressed as a series of ring domains, with the rings corresponding to segmental boundaries. The number of these rings varies across spider species, prompting the authors to infer that this rapid diversification of expression domains must underlie the diversification of spider walking leg morphologies (Turetzek et al. 2017). While these interspecific divergences of expression patterns are intriguing, these data were not accompanied by functional investigation and thus the significance of *homothorax-2* for leg patterning is not understood (but see below).

As with *homothorax*, much of the literature addressing spider gene duplicates is limited to gene expression surveys spanning well-known suites of candidate genes, such as other leg patterning transcription factors (Janssen et al. 2008; Pechmann and Prpic 2009), retinal determination network genes (Samadi et al. 2015; Schomburg et al. 2015), and Wnt family members (Janssen et al. 2021). Still, functional investigations of these genes are sparse, typically targeting individual paralogs (e.g., *sine oculis-A*; Gainett et al. 2020); undu-

plicated homologs such as *Wnt8*, *Sp6-9*, or *Distal-less* (Mcgregor et al. 2008; Pechmann et al. 2011; Setton and Sharma 2018); or entire signaling cascades, such as canonical Wnt signaling (via knockdown of *arrow*; Setton and Sharma 2021). Two works explored the function of *Six3* (*Optix*) paralogs in spiders, with one of these trialing double knockdown of both copies (Gainett et al. 2020; Schacht et al. 2020), but neither obtained a clear morphological phenotype associated with protocerebral structures (compare to Posnien and Bucher 2010).

To my knowledge, only one work has evinced the retention of function in a paralog pair in *P. tepidariorum*. In an investigation of Toll family genes, (Benton et al. 2016) discovered two copies of a Toll homolog in the genome of *P. tepidariorum*, which they designated as *Loto-A* and *Loto-B*. Single-gene RNAi experiments against each copy revealed a minor effect upon germband length when targeting *Loto-A*; RNAi against *Loto-B* did not affect the embryo. However, double knockdown of both copies simultaneously resulted in significant widening of the germband, in a manner comparable to RNAi against the single-copy homolog of *Loto* in the beetle *Tribolium castaneum*. These results suggest that the two (possibly subfunctionalized) copies of *Loto* have synergistic activities and/or that they compensate for each other in single-copy RNAi experiments. Expression levels were not reported in that work, making the exact interpretation of this double knockdown experiment elusive. Nevertheless, the feasibility of eliciting additive effects in double RNAi experiments should be explored further in future investigations of spider gene duplicates.

Thus, although neofunctionalization of ancient paralogs seems an appealing framework for explaining the evolutionary success of groups within Arachnopulmonata (specifically, spiders), functional data supporting a broad role for neofunctionalization in driving the body plan disparity of Chelicerata remain unavailable. The most evident innovations of spiders—venoms and silks—are certainly facilitated by gene duplications and expansions of venom and silk gene families, but these duplications are typically lineage-specific, comparatively young, and do not result from the ancient and shared arachnopulmonate whole genome duplication (Clarke et al. 2014, 2015; Haney et al. 2016; Chaw et al. 2021; Lüddecke et al. 2022). A clear and substantiated case of neofunctionalization in a paralog born of the arachnopulmonate genome duplication remains at large, as does the evidence that this mechanism facilitated the body plan disparity or morphological novelty in successful chelicerate groups like spiders, scorpions, and pseudoscorpions.

Why comparative data matter: two lessons from *Phalangium opilio*

The first and only model system for the study of harvestman development is the widespread, synanthropic species *Phalangium opilio*. Resources for this species include developmental transcriptomes, a draft genome, and highly penetrant embryonic RNAi (Sharma et al. 2013, 2012b; Gainett et al. 2021). In the context of whole genome duplication, *P. opilio* exhibits the essential condition required for understanding the arachnopulmonate whole genome duplication: it bears no trace of systemic gene duplication (Gainett et al. 2021). It, therefore, represents a closely related outgroup to the arachnopulmonates, analogous to the role that amphioxus has played in understanding vertebrate comparative genomics and developmental evolution (Holland et al. 2008; Putnam et al. 2008; Li et al. 2017). Over the past 11 years, *P. opilio* has served a key role in assessing the expression and function of single-copy homologs of various patterning genes, with emphasis on the evolution of appendages (Sharma et al. 2013, 2015, 2012c). In this regard, *P. opilio* offers two insights as to the cases of paralogous spider copies that were previously discussed above.

First, revisiting the case of *dachshund-2* and the patella, it is notable that the expression pattern and function of the single-copy *dachshund* homolog of *P. opilio* are known. In the harvestman, *dachshund* is initially expressed as a single ring-like domain in the developing limb buds of all three appendage types (chelicera, pedipalp, and walking leg). At later stages of leg elongation and podomere formation, *dachshund* expression spans the proximal femur through the distal patella, taking on a heterogeneous expression pattern that resembles two strong rings of expression. Comparing this homolog to the expression domains of the two spider copies (now also known to be shared with the two paralogs of scorpions and whip spiders; Gainett and Sharma 2020; Nolan et al. 2020), *dachshund* expression in *P. opilio* is analogous to the combination of *dachshund-1* (restricted to the femur) and *dachshund-2* (restricted to the patella) expression in arachnopulmonates (Fig. 5). These patterns suggest that what Turetzek et al. (2015) described as a case of neofunctionalization may in fact correspond to a subfunctionalization event—effectively splitting the femoral and patellar domains' functions across two daughter copies in the common ancestor of Arachnopulmonata.

Testing this hypothesis is made difficult by the canonical phenotype elicited by RNAi against *dachshund* in the harvestman: deletion of the femur through the patella in the pedipalps and walking legs (Sharma et al. 2013). However, one of the advantages of *P. opilio* as

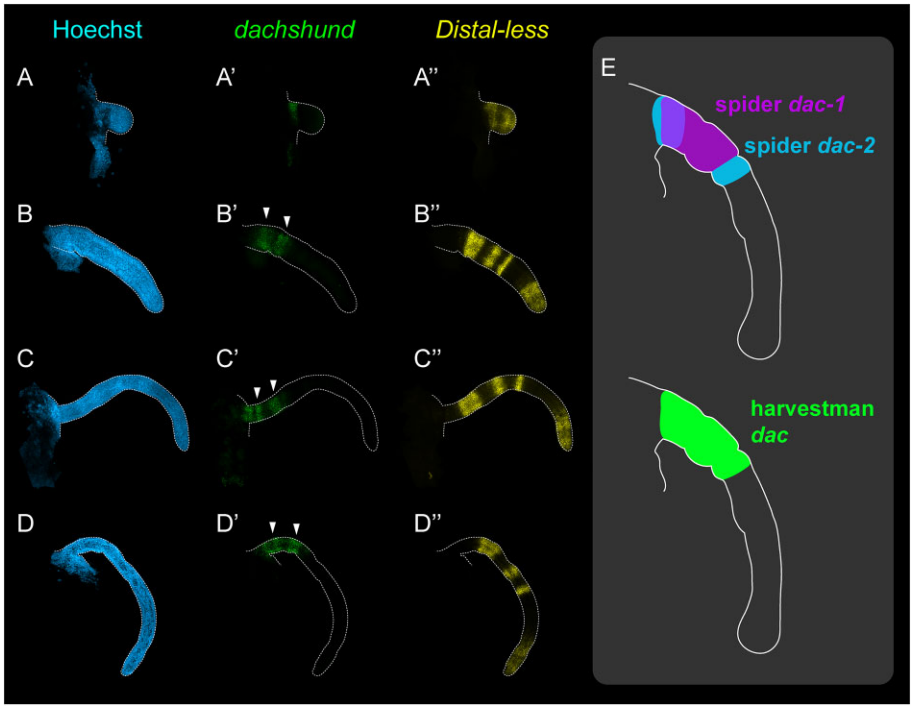

Fig. 5 Expression of *dachshund* and *Distal-less* in the second walking leg of the harvestman *P. opilio* using hybridization chain reaction. (A) Stage 9 embryo. (B) Late stage 10 embryo. (C) Stage 11 embryo. (D) Late stage 12 embryo. Note the heterogeneous expression domains of *dachshund*, corresponding to the femoral and patellar domains (arrowheads). (E) Schematic of spatial relationships of *dachshund* homologs of spiders and harvestman. Note that the domains of the spider *dachshund* paralogs reflect the subdivision of the single-copy harvestman *dachshund* homolog. Staging of embryos follows Gainett et al. (2022)

a model system is that delivery of dsRNA can be performed at various points during embryonic development (traditionally, microinjection is performed when the perivitelline space first develops, 5 days after egg laying). Targeting *dachshund* for knockdown at a later point in embryogenesis may elicit the distal patellar segmentation function that is predicted to have been retained by the single-copy homolog.

Regardless of the outcome, the dynamics of *P. opilio dachshund* underscore the importance of surveying single-copy chelicerate homologs as litmus tests for scenarios of neofunctionalization (as opposed to subfunctionalization). For an unambiguous case of neofunctionalization, the neofunctionalized arachnopulmonate copy should be expressed in a domain shared neither by its paralog nor by the outgroup's single-copy homolog. In addition, in the ideal case, three aspects of comparative development must be understood: the function of the single-copy outgroup homolog, the individual function of each duplicated ingroup copy, and the combined function of the two duplicated ingroup copies.

Second, revisiting the Hox genes, successful RNAi experiments against spider Hox genes remain limited, and many single-paralog knockdowns do not elicit phenotypes (e.g., *labial-2*; Pechmann et al. 2015). By contrast, RNAi against single-copy homologs of *P. opilio* have yielded phenotypes for genes that could not be interrogated in spiders (e.g., *homothorax*; *Sex combs reduced*; Sharma et al. 2015; Gainett et al. 2021), possibly because these genes are paralogous in spiders and exhibit compensatory effects in RNAi experiments. Furthermore, other RNAi experiments in the harvestman have resulted in entirely different phenotypic spectra that provide new insights into the patterning of the chelicerate body plan. As an example, knockdown of *labial-1* in *P. tepidariorum* resulted in the deletion of the pedipalpal segment altogether, sometimes in addition to the first walking leg (L1) segment; this phenotype was understood to imply a role of *labial-1* in the maintenance of the pedipalpal (and possibly also L1) territory (Pechmann et al. 2015). This data point did not elicit a homeotic function for *labial-1*, leaving doubt as to how pedipalpal identity is conferred. By contrast, RNAi against the single-copy *labial* homolog of the harvestman resulted in homeotic transformation of pedipalps into chelicerae, in addition to phenotypes that reflected the

segmental maintenance function previously observed in spiders (Gainett et al. 2023). This result constituted the first known case of canonical Hox function in a *labial* homolog in Arthropoda (i.e., tritocerebral to deutocerebral homeosis).

These outcomes highlight the need for comparative studies of the same genes in taxa that exhibit the duplication, as well as outgroup taxa that do not. Given the evolutionary lability of Hox function across expression domains (particularly the fluid posterior boundaries; Sharma et al. 2012b; Damen et al. 1998; Telford and Thomas 1998), the same experiments may yield markedly different outcomes and insights across different chelicerate taxa.

Developmental system drift: an overlooked null hypothesis for duplication dynamics

While historically informative, traditional single-channel gene expression surveys of candidate genes using colorimetric reagents are being superseded by multichannel fluorescent expression assays via hybridization chain reaction, as well as high throughput, single-cell RNA-Seq (scRNA-Seq) datasets, which offer greater precision and accuracy in characterizing the dynamics of paralogous genes and accelerate lineage-specific gene discovery (Choi et al. 2018; Akiyama-Oda et al. 2022; Leite et al. 2022). One approach to identifying putative cases of sub- and neofunctionalization in scRNA-Seq data is to look for distinct populations of cells expressing each of the paralogs; the spatiotemporal divergence of expression levels could be taken as a potential case of divergence of gene function across daughter copies (Leite et al. 2022).

Although this approach to interpreting divergence of gene function is promising, one potential pitfall is that distinct expression levels could also reflect cases of developmental system drift, wherein homologous structures are patterned by non-homologous developmental mechanisms (True and Haag 2001). Classic examples of developmental system drift in arthropods includes anteroposterior segmentation; the segments of arthropods are unambiguously understood to reflect a trait resulting from common ancestry, but there are major differences in segmentation processes across the arthropod tree of life. These differences span the mode of segment formation (sequential addition from a posterior growth zone versus simultaneous formation of all segments), as well as the signaling cascades involved (Wnt and Notch–Delta signalling versus the canonical segmentation cascade of long-germ embryogenesis in the fruit fly *D. melanogaster*) (Peel et al. 2005; Damen 2007; Clark 2017).

In spiders, an example was provided above for variation in *homothorax-2* expression domains across spider species (Turetzek et al. 2017)—the authors inferred that the *homothorax* duplicates were spider-specific and that these differences in expression domains were adaptive (e.g., possibly associated with variation in spider leg morphology). However, in rapid succession, it was discovered that *homothorax* was duplicated in the common ancestor of Arachnopulmonata as a result of the arachnopulmonate whole genome duplication (Schwager et al. 2017; Leite et al. 2018); and that *homothorax-1 and homothorax-2* were similarly expressed in scorpions and whip spiders (Gainett and Sharma 2020; Nolan et al. 2020). Thus, the divergence of *homothorax* paralogs' expression domains is not a spider-specific phenomenon (Fig. 4).

Associating the divergence of the arachnopulmonate *homothorax* copies' spatiotemporal domains with adaptive changes is not straightforward, owing both to the paucity of available data points and to the lack of functional data for either arachnopulmonate paralog. It is tempting to infer that *homothorax-1* (which exhibits the conserved expression pattern) retains the conserved function, whereas *homothorax-2* has acquired novel domains, possibly relating to leg segmentation or differential elongation of specific podomeres (Nolan et al. 2020). However, the sole functional data point for a chelicerate *homothorax* homolog suggests that both roles may have been present in the chelicerate common ancestor. Knockdown of the single-copy homolog of *homothorax* in the harvestman *P. opilio* resulted in a wide phenotypic spectrum that included segmental fusions, defects of proximal appendage segments, and homeosis of anterior appendages, paralleling experimental data for *homothorax* homologs in insect models (Ronco et al. 2008; Sharma et al. 2015). Part of the harvestman phenotypic spectrum included disruption of proximal leg segments and defects in podomere boundaries in affected walking legs (Fig. 4i of Sharma et al. 2015)—again, paralleling outcomes of RNAi against insect homologs of *homothorax* (Angelini and Kaufman 2004; Ronco et al. 2008). Thus, it is possible that the single-copy homolog of *homothorax* plays a conserved role in proper formation of podomere boundaries in addition to its canonical roles as a Hox cofactor and proximo-distal axis-patterning gene (a side activity, *sensu* Bergthorsson et al. 2007). Subsequent to the duplication, selection for increased dosage may have maintained the *homothorax-2* paralog, followed by mutational improvement of the extra copy and gradual segregation of the side activity entirely in the "new" paralog. This inference is partially supported by the expression domains of *homothorax* paralogs of the whip spider *Phrynus marginemac-*

ulatus, wherein *homothorax-2* is expressed only as two domains in the leg (one proximal and the other subtending the presumptive tarsus) whereas *homothorax-1* is expressed heterogeneously, both as a continuous band through most of the developing leg, as well as stronger rings of expression corresponding to segmental boundaries (Gainett and Sharma 2020) (Fig. 4). The differences in expression domains in the three surveyed arachnopulmonate orders may not reflect acquisition of novel functions, so much as divergent partitioning of the ancestral functions between the two available *homothorax* copies.

Alternatively, changes in the expression domains of *homothorax-2* across the surveyed Arachnopulmonata may not be adaptive or even functional, but rather, could reflect drift of functionally redundant domains in the "new" paralog, which may be slated for eventual gene loss (Lynch and Conery 2000; Johri et al. 2022). Similar dynamics have been described for *nubbin* expression across arthropods, and particularly insects, with little correlation between expression patterns and ensuing leg morphologies (Li and Popadic 2004; Prpic and Damen 2005; Hrycaj et al. 2008; Turchyn et al. 2011), as with chelicerate *homothorax* patterns.

Developmental system drift should be considered a valid and valuable null hypothesis in the analysis of diverging gene expression patterns in Chelicerata, though it is rarely invoked in the chelicerate literature. Given the intuitive link between divergence of duplicated genes and evolutionary novelty, whole genome duplications frequently impel searches for cases of sub- and neofunctionalization of paralogs, and the spider developmental literature is no exception. Yet, approaching the evolution of ancient spider paralogs with the expectation of finding cases of neofunctionalization may obscure broader and more prevalent dynamics across arachnopulmonate body plan evolution, such as drift, swapping of functions with non-homologous genes, and cooption of ancient gene cassettes to serve new functions (Li and Popadic 2004; Pechmann et al. 2011; Setton and Sharma 2018).

Conclusion

Despite the allure of whole genome duplication as an explanatory vehicle for evolutionary success, diversification dynamics of the extant chelicerates do not substantiate a direct correlation between incidence of duplication and species richness. A review of available literature shows that few functional datasets exist that inform the divergence of functions in paralogous gene pairs in the spider *P. tepidariorum*. Of that handful of existing datasets, none support the inference that neofunctionalization of ancient paralogs has played a significant role in spider (or arachnopulmonate) body plan evolution. Definitive identification of neofunctionalization requires comparative functional data from non-arachnopulmonate groups such as *P. opilio*, which can serve to polarize the evolution of gene expression patterns, as well as comparative expression data from satellite models (both arachnopulmonate and non-arachnopulmonate) for testing the explanatory power of evolutionary scenarios more generally. In future, such combined investigations of duplicated and single-copy homologs across chelicerates may serve to pinpoint whether and how neofunctionalization of ancient paralogs stemming from genome duplication have shaped the evolution of Chelicerata.

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Conflict of Interest

No conflicts to declare.

Data Availability

All data reviewed in this article have been made public previously.

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