Arthropod Phylotranscriptomics With a Special Focus on the Basal Phylogeny of the Myriapoda

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Abstract

Arthropoda represents the most diverse animal phylum, but clarifying the phylogenetic relationships among arthropod taxa remains challenging given the numerous arthropod lineages that diverged over a short period of time. In order to resolve the most controversial aspects of deep arthropod phylogeny, focusing on the Myriapoda, we conducted phylogenetic analyses based on ten super-matrices comprised of 751 to 1,233 orthologous genes across 64 representative arthropod species, including 28 transcriptomes that were newly generated in this study. Our findings provide unambiguous support for the monophyly of the higher arthropod taxa, Chelicerata, Mandibulata, Myriapoda, Pancrustacea, and Hexapoda, while the Crustacea are paraphyletic, with the class Remipedia supported as the lineage most closely related to hexapods. Within the Hexapoda, our results largely affirm previously proposed phylogenetic relationships among deep hexapod lineages, except that the Paraneoptera (Hemiptera, Thysanoptera, and Psocodea) was recovered as a monophyletic lineage in some analyses. The results corroborated the recently proposed phylogenetic framework of the four myriapod classes, wherein Symphyla and Pauropoda, as well as Chilopoda and Diplopoda, are each proposed to be sister taxa. The findings provide important insights into understanding the phylogeny and evolution of arthropods.

Key words: arthropods, myriapods, transcriptome, phylogenetics, evolution.

Significance

This study elucidates the phylogenetic relationships of arthropods using multiple super-matrices of transcriptome sequence data, with comprehensive and well-balanced sampling across the phylum Arthropoda. Arthropoda represents the most diverse animal phylum, but clarifying the phylogenetic relationships among arthropod taxa remains challenging given the numerous arthropod lineages that diverged over a short period of time. Although phylogenomics has emerged as an increasingly powerful tool for resolving challenging phylogenetic questions, datasets constructed using different samples or different gene loci occasionally generate contradictory results. In order to resolve the most controversial aspects of deep arthropod phylogeny, we conducted phylogenetic analyses based on ten super-matrices comprised of 751 to 1,233 orthologous genes across 64 representative arthropod species, including 28 transcriptomes that were newly generated in this study, followed by the identification of the consensus topologies that were supported by all of the resulting trees. Our results provide unambiguous support for the monophyly of the higher arthropod taxa, Chelicerata, Mandibulata, Myriapoda, Pancrustacea, and Hexapoda, while the Crustacea are paraphyletic, with the class Remipedia supported as the lineage most closely related to hexapods. The results also corroborated the recently proposed phylogenetic framework of the four myriapod classes, wherein Symphyla and Pauropoda, as well as Chilopoda and Diplopoda, are each proposed to be sister taxa. Within the Hexapoda, our results largely affirm previously proposed phylogenetic relationships among deep hexapod lineages, but the Paraneoptera (Hemiptera, Thysanoptera, and Psocodea) was recovered as a monophyletic lineage in some analyses. These findings provide important insights into understanding the phylogeny and evolution of arthropods.

Introduction

Of the ~1.2 million animal species that have been described to date, about 80% are arthropods (DeSalle and Schierwater 2021). The phylum Arthropoda is thus the most diverse animal group on earth, and the group has undergone considerable morphological, ecological, and functional diversification. Consequently, as a taxonomic group, arthropods are well suited to studies on the evolution and diversification of living organisms. On the other hand, the extremely high level of species diversity of arthropods, with their deep lineages that have diverged over a short period of time as suggested by the fossil record of the early Cambrian and fossil-calibrated time trees (e.g. Lee et al. 2013), makes it difficult to elucidate their phylogenetic relationships, the most fundamental information for understanding arthropod evolution.

Extant arthropods consist of four higher-level taxonomic groups, Chelicerata (sea spiders, horseshoe crabs, spiders, scorpions, and their relatives), Myriapoda (centipedes, millipedes, garden centipedes, and pauropods), the traditional Crustacea (decapods, seed shrimps, fish lice, krill, isopods, barnacles, copepods, amphipods, mantis shrimps, branchiopods, horseshoe crabs, and remipedes), and Hexapoda (insects). For more than a century up to the mid-1990s, it was steadfastly believed that, of the four arthropod groups, the myriapods and hexapods were sister groups; that is, that the two groups diverged from a common ancestor and have terrestrial origins. This traditional view was proposed based on morphological examinations of extant arthropods and their fossils. As primary evidence, tracheal breathing and the degeneration of the second pair of antennae are common to both myriapods and hexapods, so these two groups were classified into the traditional taxa Tracheata (Haeckel 1866; Pocock 1893a, 1893b) and Atelocerata (Heymons 1901). In the mid-1990s, molecular phylogenetic analyses revolutionized our traditional understanding of arthropod phylogeny and evolution. Friedrich and Tautz (1995) were the first to challenge traditional ideas about the phylogenetic relationships among the four major arthropod groups. Their study using ribosomal DNA rejected the sister-relationship between hexapods and myriapods, and suggested that hexapods are closely related to crustaceans. Since then, numerous studies have been conducted to test this hypothesis using a variety of molecular data, including nuclear and mitochondrial DNA (Boore et al. 1998; Hwang et al. 2001; Hassanin 2006), several to hundreds of genes (Shultz and Regier 2000; Cook et al. 2001; Giribet et al. 2001; Mallatt et al. 2004; Mallatt and Giribet 2006; Regier et al. 2008, 2010; von Reumont et al. 2009; Koenemann et al. 2010; Meusemann et al. 2010; Rota-Stabelli et al. 2011; Zwick et al. 2012; Noah et al. 2020; Szucsich et al. 2020), and whole genome sequences (Thomas et al. 2020; see reviews Edgecombe 2010; Giribet and Edgecombe 2012, 2019). All of these studies support the hypothesis that hexapods and crustaceans belong to the same clade, now referred to as Pancrustacea (Giribet et al. 2001; Giribet and Edgecombe 2019). On the other hand, the phylogenetic placement of myriapods within arthropods remains somewhat controversial, i.e. the Mandibulata (Myriapoda + Pancrustacea) vs. the Myriochelata or Paradoxopoda (Myriapoda + Chelicerata) (Giribet and Edgecombe 2013). The Myriochelata hypothesis, which places the myriapods with the chelicerates, has been supported by some molecular phylogenetic analyses (see Giribet and Edgecombe 2013), mitogenome studies of gene order (Dong et al. 2012a, 2012b), and developmental and gene expression data (Kadner and Stollewerk 2004; Mayer and Whitington 2009). In recent years, however, numerous molecular phylogenetic studies (e.g. Regier et al. 2010; Leite and McGregor 2016; Giribet and Edgecombe 2019; Noah et al. 2020; Thomas et al. 2020) together with morphological and fossil evidence (Legg et al. 2013; Edgecombe 2017; Maurer et al. 2019) support the monophyly of Mandibulata with Chelicerata as a sister group.

All extant taxa within the Myriapoda are terrestrial and are classified into four classes: Chilopoda (centipedes), Diplopoda (millipedes), Pauropoda (pauropods), and Symphyla (symphylans). The Chilopoda, which is the only wholly predatory myriapod group, comprises ~5,000 known species (Brewer et al. 2012). The Diplopoda is the most diverse myriapod group, with 16 extant orders and more than 13,500 identified species (Brewer et al. 2012; MilliBase: https://millibase.org/). This group is characterized by having diplosegments, where each body segment supports two pairs of legs. The members of the remaining two classes, Pauropoda and Symphyla, are small, translucent, soil-dwelling myriapods, with body lengths measuring < 2 mm and mostly 1 to 8 mm (but exceptionally up to 13 mm), respectively. To date, 835 pauropod species across 2 orders and 5 families, and 195 Symphylid species in one order and two families have been described (Scheller 2011; Szucsich and Scheller 2011). Although the monophyly of each class was supported by both morphological and molecular evidence (Dohle 1980; Regier 2005a, 2005b), a variety of hypotheses have been proposed regarding the phylogenetic relationships among the classes (Fig. 1, see Edgecombe and Giribet 2002, Fig. 6.1). For example, numerous morphological and developmental studies have consistently shown that Pauropoda and Diplopoda are sister lineages; as a result, these two classes have been grouped together as Dignatha (Pocock 1893a, 1893b; Tiegs 1947; Dohle 1980; Edgecombe et al. 2000). Symphyla and Dignatha have traditionally been classified into the taxon Progoneata (Fig. 1a) (Pocock 1893a, 1893b; Dohle 1980; Edgecombe et al. 2000), but an affinity between Symphyla and Chilopoda has also been suggested based on the structure of the second maxilla (Fig. 1b) (Tiegs 1947). The Progoneata–Dignatha hypothesis (Fig. 1a) is also

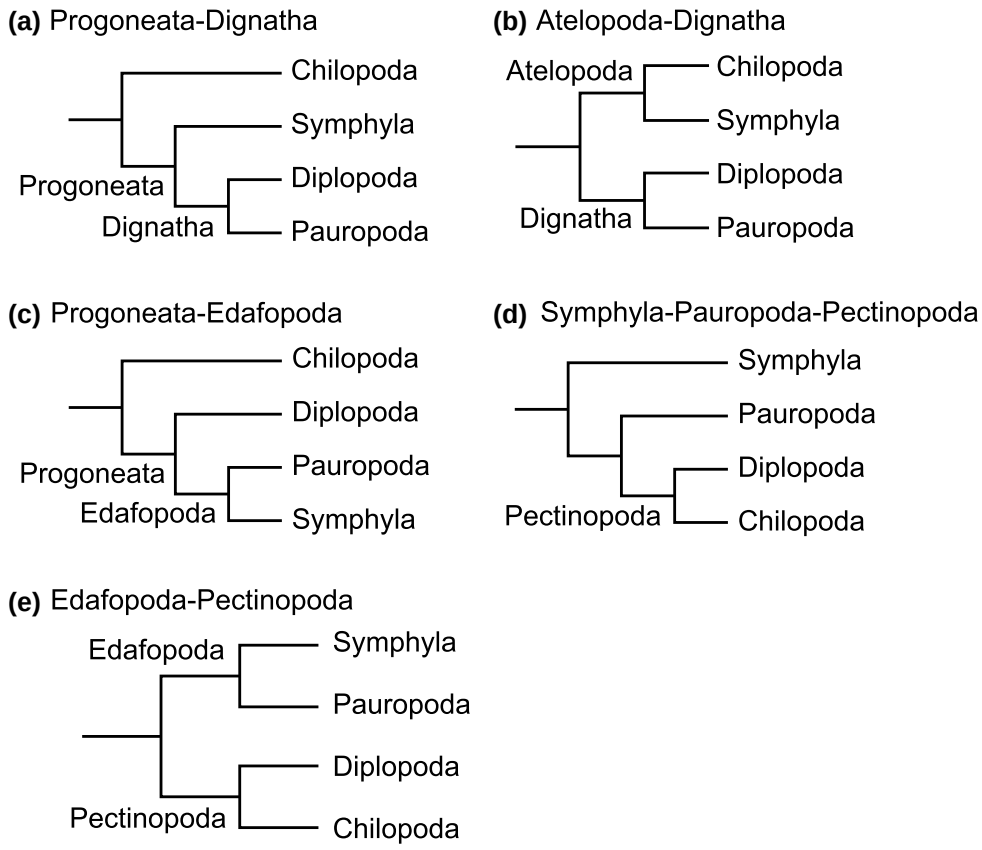

Fig. 1. Major hypotheses for phylogenetic relationships among the four myriapod classes proposed to date. a, b) Traditional views based on morphology (Pocock 1893a, 1893b; Tiegs 1947; Fernández et al. 2018) and supported by some molecular analyses (Fernández et al. 2018). c, d) Hypotheses based primarily on molecular analyses (Regier et al. 2010; Dong et al. 2012a, 2012b; Zwick et al. 2012; Miyazawa et al. 2014; Rehm et al. 2014; Noah et al. 2020). e) Hypothesis based on Szucsich et al. (2020), Wang et al. (2021), Benavides et al. (2023), and this study.

supported by recent molecular studies (Fernández et al. 2018; Zuo et al. 2022). In contrast to this traditional view, three alternate hypotheses (Fig. 1c to e) have also been proposed based on molecular phylogenetic analyses (Gai et al. 2006; Regier et al. 2010; Dong et al. 2012a, 2012b; Zwick et al. 2012; Miyazawa et al. 2014; Rehm et al. 2014; Noah et al. 2020; Szucsich et al. 2020; Wang et al. 2021; Benavides et al. 2023).

As described above, strong support exists for the crustaceans and hexapods belonging to a common group, Pancrustacea, with crustaceans being paraphyletic and hexapods diverging from a lineage of crustaceans. Phylogenetic relationships among higher hexapod taxa, including at the ordinal level, are comparatively well understood (Ishiwata et al. 2011; Misof et al. 2014). However, despite the efforts of numerous researchers, the phylogenetic relationships between higher crustacean taxa are less well resolved, except that Oligostraca is considered to be the sister group to all other pancrustaceans (Regier 2005a, 2005b; von Reumont et al. 2012; Rota-Stabelli et al. 2013; Schwentner et al. 2018; Lozano-Fernandez et al. 2019a, 2019b; Bernot et al. 2023; Yu et al. 2024). In particular, recent studies based on large amounts of molecular data have yielded different results concerning the crustacean lineages that are most closely related to hexapods. For example, two studies focusing on 62 protein-coding genes proposed that the hexapods diverged from a common ancestral lineage of the Remipedia and Cephalocarida, named Xenocarida (Regier et al. 2010; Noah et al. 2020). In contrast, the findings of transcriptome sequence studies showed that the Remipedia are closely related to hexapods (Schwentner et al. 2018; Lozano-Fernandez et al. 2019a, 2019b; Bernot et al. 2023; Yu et al. 2024). Yu et al. (2024) further suggested that the close relationship between Remipedia and Cephalocarida is due to systematic errors and proposed a new grouping of Copepoda + Allotriocarida (Branchiopoda, Cephalocarida, Remipedia, and Hexapoda), which is consistent with the results of Bernot et al. (2023).

Thus, the deep phylogenetic relationships of arthropods remain obscure or controversial, and the ambiguity in phylogenetic relationships hinders our understanding of the origin and evolution of arthropods. Recently, phylogenomics, which uses genome-scale data to infer phylogenetic relationships, has emerged as an increasingly powerful tool for resolving challenging phylogenetic questions. However, datasets constructed using different samples or

different gene loci occasionally generate contradictory results, despite high bootstrap values and/or posterior probabilities supporting the resultant phylogenetic tree topologies (Regier et al. 2010; Rehm et al. 2014; Fernández et al. 2016, 2018; Schwentner et al. 2018; Noah et al. 2020; Szucsich et al. 2020; Bernot et al. 2023; Yu et al. 2024). One of the most likely reasons for these disparities is that the identification of orthologous genes is not perfect (Cheon et al. 2020), which implies that the datasets contain some genes that do not reflect true phylogenetic relationships. Although a variety of software programs are publicly available, the accurate identification of orthologous genes and the complete removal of genes that are inappropriate for phylogenetic analysis is extremely difficult. Therefore, in this study we adopted an approach that involved the creation of multiple super-matrices using different sequence data and samples to perform phylogenetic analysis, followed by the identification of the consensus topologies that were supported by all of the resulting trees. Finally, we conducted phylogenetic analyses based on ten super-matrices, which contained 751 to 1,233 orthologous genes from 64 representative arthropod species, of which 28 transcriptomes are newly generated in this study. The results provided strong support for numerous deep phylogenetic nodes for arthropods, especially the phylogenetic relationships among the myriapod classes.

Results

Datasets

Figure 2 shows the workflow employed for data matrix compilation and tree construction. The number of reads, contigs, and orthologous genes for each sample are shown in supplementary table S1, Supplementary Material online). The orthologous genes of each sample were identified from transcriptomes based on two core orthologous gene sets, insecta_hmmer3_2 (Coreset A in this study) and arthropoda_hmmer3 (Coreset B in this study), respectively (Fig. 2) (Ebersberger et al. 2009). The orthologous genes identified based on Coreset A and Coreset B were then used to compile sequence alignments with five sample sets, as described in the "Materials and Methods" section. As a result, a total of ten sequence datasets, Datasets A1 to A5 and Datasets B1 to B5, were compiled and used for phylogenetic analyses (Fig. 2). The final super-matrices of Datasets A1 to A5 had 1,077 to 1,234 orthologous genes and 251,310 to 333,717 amino acids (aa), and the percentages of average missing data were 6.07 to 9.07 for orthologs and 9.8 to 11.54 for aa (Table 1). For the Datasets B1 to B5, those were 751 to 841, 187,693 to 251,262, 5.84 to 7.51, 8.84 to 10.79, respectively (Table 1). The detailed data of the samples in each dataset are presented in supplementary tables S2 to S11, Supplementary Material online.

Tree Construction and Topologies

Each super-matrix of Datasets A1 to A5 and Datasets B1 to B5 was used for phylogenetic analysis with RAxML and MrBayes, respectively, except for Dataset A1 and Dataset B1, which were only subjected to RAxML analysis (see "Materials and Methods" section). The resulting trees are shown in supplementary figs. S1 to S11, Supplementary Material online. Two RAxML trees that were inferred based on the super-matrices, Dataset A1 and Dataset B1, which contain all the samples used in this study, are shown in Figs. 3 and 4. The bootstrap and posterior probability supports for tree topologies constructed based on other datasets are also indicated in these two trees (Figs. 3 and 4).

Comparison of the inferred trees based on Datasets A1 to A5 showed no differences on the tree topology between the RAxML and MrBayes analyses (Fig. 3 and supplementary figs. S1 to S5, Supplementary Material online). Between the datasets, however, there were some differences in the tree topology for the phylogenetic positions of the three species, one acarid species (*Ixodes scapularis*), and two myriapod species (*Diplomaragna* sp. and *Cryptocorypha* sp.) (Fig. 3). Datasets A1 to A3 showed that *I. scapularis* was clustered with the class Merostomata (supplementary figs. S1 to S3, Supplementary Material online), but Dataset A5 included the Acarid species into the class Arachnida (supplementary fig. S5, Supplementary Material online). In addition, the phylogenetic positions of the two myriapod species were switched among the Datasets A1, A3, and A4 (Fig. 3 and supplementary figs. S1, S3 and S4, Supplementary Material online). On the other hand, no differences in the tree topologies were observed among Datasets B1 to B5 (Fig. 4 and supplementary figs. S6 to S11, Supplementary Material online), which were compiled with the orthologous genes identified based on Coreset B, while marked differences in the tree topologies derived for the Crustacea were observed between the RAxML and MrBayes analyses (Fig. 3 and supplementary figs. S10 and S11, Supplementary Material online).

Discussion

Phylogenetic Relationships Among the Basal Lineages of Arthropods

Figure 5 shows the phylogenetic relationships among arthropods that are supported by all trees inferred using different datasets and methods. The results unambiguously support the monophyly of the Chelicerata, Mandibulata, Myriapoda, Pancrustacea, and Hexapoda, and confirmed that extant arthropods first divided into two large groups, the Chelicerata and Mandibulata (Myriapoda + Pancrustacea) (Figs. 3 to 5). The traditional taxon of the subphylum Crustacea as paraphyletic, and its association with

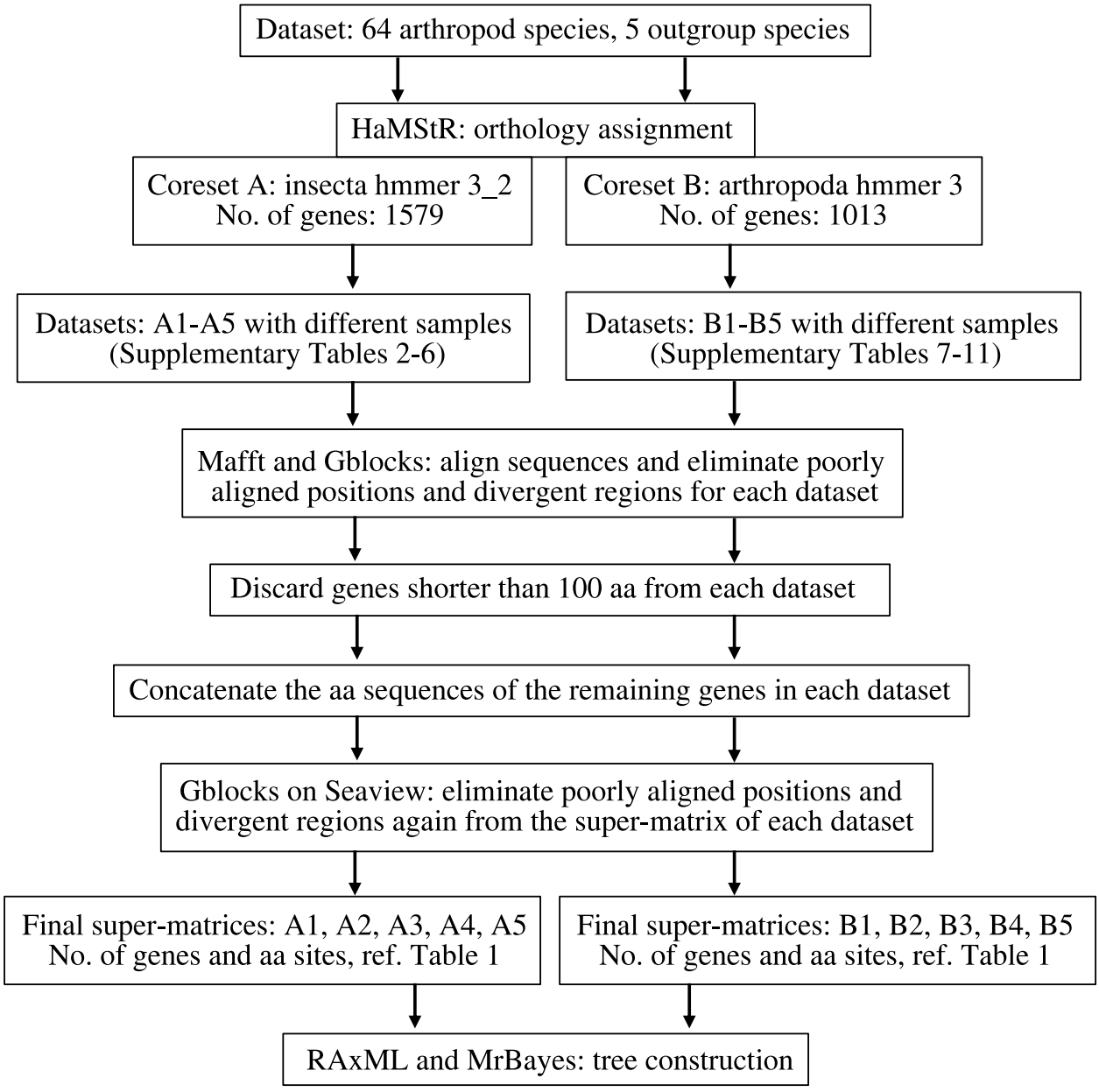

Fig. 2. Workflow showing data matrix compilation and the tree reconstruction.

the Hexapoda to form the monophyletic group Pancrustacea, is supported by this study (Fig. 5). These results are consistent with numerous previous molecular phylogenetic analyses (e.g. Regier et al. 2010; Noah et al. 2020; Giribet and Edgecombe 2019). Although the Myriochelata (or Paradoxopoda) hypothesis, which suggests a sister-relationship between Myriapoda and Chelicerata, has been proposed and supported by some molecular phylogenetic analyses (Hwang et al. 2001; Mallatt et al. 2004; Pisani et al. 2004; Dunn et al. 2008), evidence from the gene order of mitogenomes (Dong et al. 2012a, 2012b) and limited morphological evidence, such as neurogenesis similarities (Kadner and Stollewerk 2004) and development in velvet worms (Mayer and Whitington 2009), the consensus of most molecular phylogenetic studies (e.g. Regier et al. 2010; Leite and McGregor 2016; Giribet and Edgecombe 2019; Noah et al. 2020; Thomas et al. 2020) and morphological evidence, including fossil records (Legg et al. 2013; Edgecombe 2017; Maurer et al. 2019) refute the Myriochelata hypothesis in favor of monophyly of Mandibulata. The present study also provides no evidence in support of the Myriochelata hypothesis (Figs. 3 and 4 and supplementary figs. S1, S2, S6 and S7, Supplementary Material online). Notably, recent studies

Table 1 Data information of super-matrices for phylogenetic analyses

Datasets	Coresets for HaMSrR	Sample sets	Super-matrices			Super-matrices (eliminated genes shorter than 100 aa)			
			Orthologs	Average missing (%)	Amino acids (aa)	Average missing (%)	Orthologs	Average missing (%)	Amino acids (aa)	Average missing (%)
Dataset A1	Coreset A (insecta hmmer 3_2)	Arthropods (62) + Outgroups (3)	1358	5.53	268819	10.00	1077	9.07	251310	10.75
Dataset A2		Arthropods (31) + Outgroups (2)	1452	4.67	333080	9.15	1233	7.85	318911	9.8
Dataset A3		Myriapods (20) + Outgroups (15)	1457	4.68	341608	9.94	1226	7.99	327013	10.66
Dataset A4		Myriapods (18)	1394	3.06	324205	10.67	1166	6.07	310673	11.54
Dataset A5		Pancrustaceans (33) + Outgroups (8)	1442	4.4	347277	9.29	1234	7.19	333717	9.84
Dataset B1	Coreset B (arthropoda hmmer 3)	Arthropods (62) + Outgroups (4)	922	6.19	196917	8.94	751	7.51	187693	9.65
Dataset B2		Arthropods (30) + Outgroups (4)	969	5.64	248673	8.87	837	6.39	241198	9.39
Dataset B3		Myriapods (20) + Outgroups (14)	949	3.58	247327	9.37	830	6.91	239793	10.01
Dataset B4		Myriapods (18)	935	2.89	232181	9.96	798	5.98	224473	10.79
Dataset B5		Pancrustaceans (34) + Outgroups (7)	948	3.29	258591	8.41	841	5.84	251262	8.84

Datasets A1 and B1, all samples; Datasets A2 and B2, focus on phylogenetic analysis at subphylum level; Datasets A3 and B3, focus on Myriapoda; Datasets A4 and B5, focus on phylogenetic analysis omong orders of two myriapod classes Chilopoda and Diplopoda; Datasets A5 and B5, focus on Pancrustacea. For the detailed information of each dataset, refer to supplementary tables S2 to S11.

on gene gain and loss patterns across genomes suggest a closer phylogenetic proximity between Myriapoda and Chelicerata than to Pancrustacea (So et al. 2022).

Within the Chelicerata, the class Pycnogonida (sea spiders) was previously suggested to be the outgroup of all other arthropods, based on phylogenetic analyses incorporating molecular sequences and morphological data, thereby challenging the monophyly of the Chelicerata (Giribet et al. 2001). However, numerous subsequent studies have provided robust and strong support for the monophyly of chelicerates, including sea spiders (Regier et al. 2010; Rehm et al. 2014; Noah et al. 2020; Giribet and Edgecombe 2019; Edgecombe 2020). The comprehensive phylogenetic analyses conducted in the present study also provide strong support for the monophyly of Chelicerata, with Pycnogonida as an outgroup relative to the analyzed euchelicerates (Xiphosura, Parasitiformes, Opiliones, and Araneae) (Figs. 3 and 4 and supplementary figs. S1, S2, S6 and S7, Supplementary Material online). However, the three arachnids were not grouped into a single clade supporting the monophyly of Arachnida, and one of them, *I. scapularis* (Parasitiformes) was shown to be more closely related to the horseshoe crabs (Xiphosura) than to the other two arachnids, i.e. *Pseudogagrella* sp. (Opiliones) and *Parasteatoda tepidariorum* (Araneae) (Fig. 3). Despite employing a great variety of analyses and a large number of molecular and morphological data, numerous studies have not managed to clarify the phylogenetic relationships among the chelicerates (Sharma et al. 2014, 2021; Ballesteros and Sharma 2019; Giribet and Edgecombe 2019; Ballesteros et al. 2022). The main reasons are considered to be due to their ancient rapid radiation, the incidence of elevated evolutionary rates in several lineages, and morphological convergence resulting from adaptations to life in terrestrial habitats (Ballesteros et al. 2022). The results of numerous molecular phylogenetic studies do not support the monophyly of Arachnida and suggest that the arachnids are paraphyletic to horseshoe crabs (Sharma et al. 2014, 2021; Ballesteros and Sharma 2019; Ballesteros et al. 2022). However, it is possible that future increases in the number of chelicerate species sampled in genomic-scale datasets may support monophyly in the arachnids (Lozano-Fernandez et al. 2019a, 2019b). Despite the limited sampling, the present results suggest that Xiphosura is an outgroup of Opiliones and Araneae (Figs. 3 and 4), and may provide insights into understanding the phylogeny and evolution of the chelicerates.

Phylogenetic Relationships of Myriapods

The myriapods have been classified into four taxonomic classes: Chilopoda, Symphyla, Diplopoda, and Pauropoda. The phylogenetic relationships among these four large groups are very confusing, and previous phylogenetic

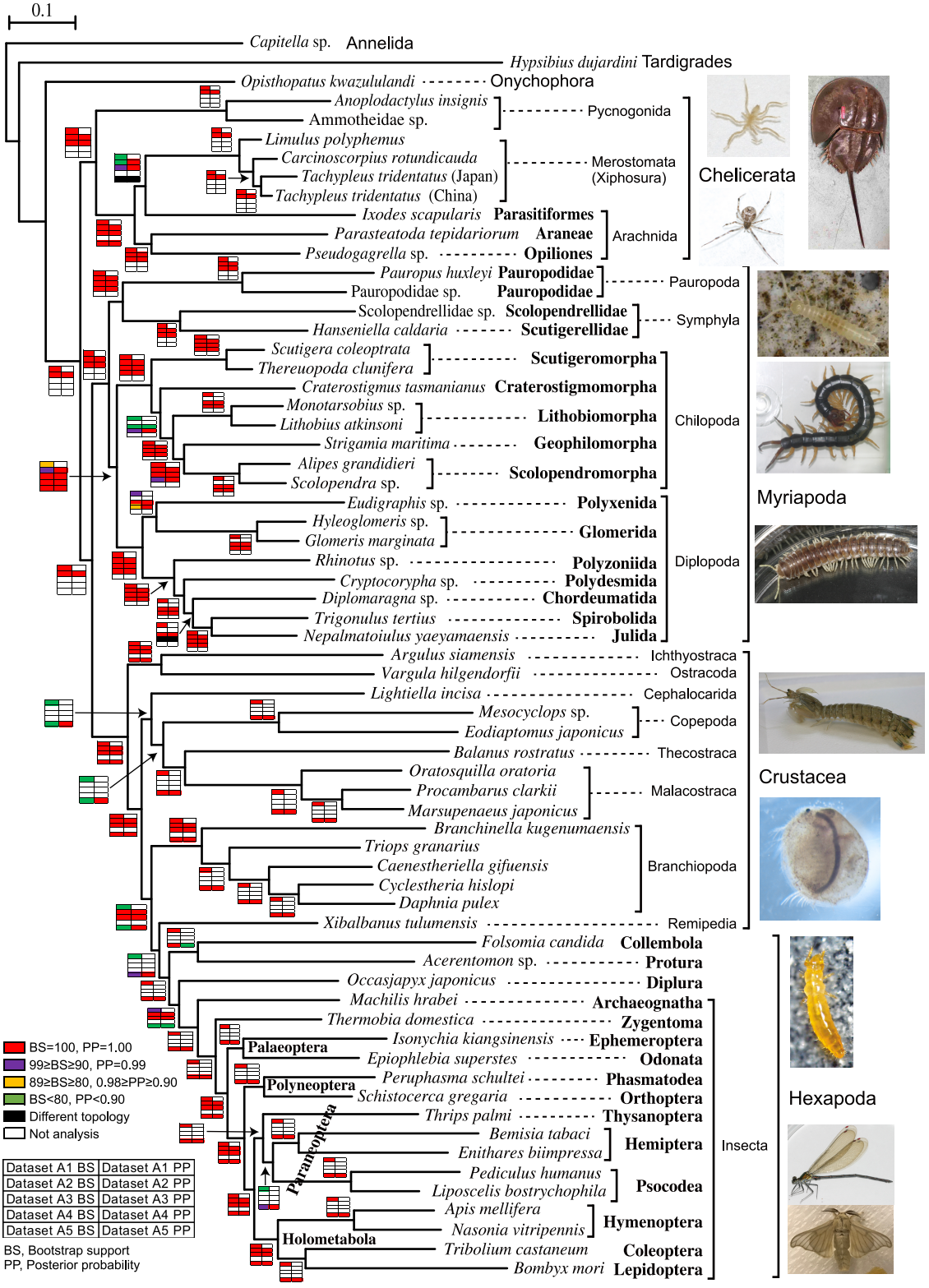

Fig. 3. RAxML tree derived from Dataset A1, which contains all arthropod species analyzed in this study. BS values and PP for tree topologies inferred from Dataset A1 and four other datasets (Datasets A2 to A5) are displayed at the tree nodes. These datasets were compiled using the core orthologous gene set, insecta_hmmer3_2 (Ebersberger et al. 2009) (Coreset a) and different sample sets (refer to supplementary tables S2 to S6, Supplementary Material online). For the trees inferred based on these datasets (see supplementary figs. S1 to S5, Supplementary Material online).

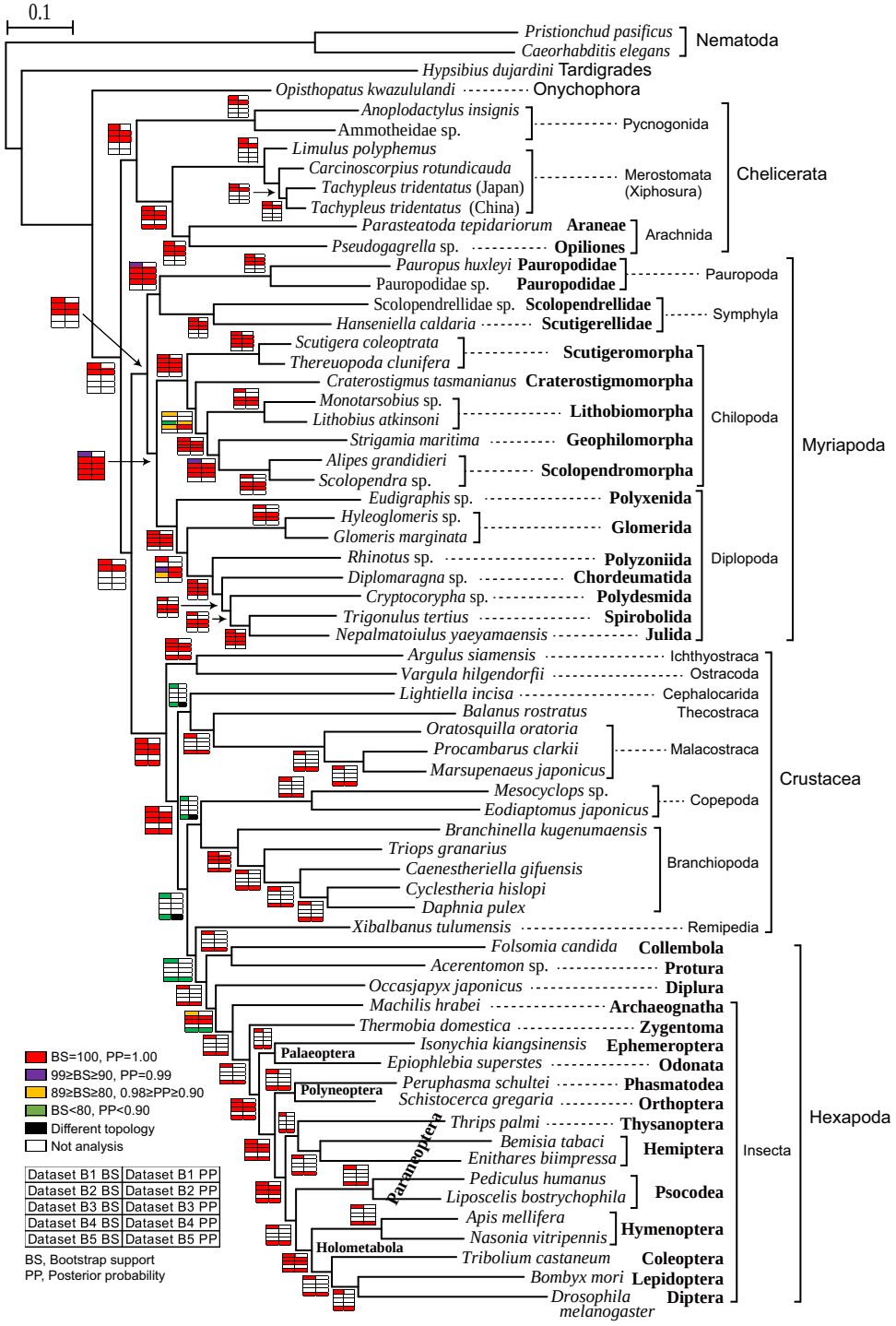

Fig. 4. RAxML tree based on Dataset B1 containing all of the arthropod species used in this study. BS and PP for the tree topologies, derived from Dataset B1 and four other datasets (Datasets B2 to B5), are merged and displayed at the tree nodes. These datasets are compiled based on the core orthologous gene set, arthropoda_hmmer3 (Ebersberger et al. 2009) (Coreset B), incorporating various sample sets (see supplementary tables S7 to S11, Supplementary Material online). For the trees resulting from these datasets (see supplementary figs. S6 to S11, Supplementary Material online).

studies based on molecular and morphological evidence have proposed a number of possible hypotheses (see Fig. 1, also Edgecombe and Giribet 2002, Fig. 6.1). The Progoneata–Dignatha (Fig. 1a) and Atelopoda–Dignatha (Fig. 1b) hypotheses, which are based primarily on morphological characters such as the anterior position of the gonopore, the fusion of the labrum, the presence of a swollen trichobothria with a basal bulb, and a limbless second

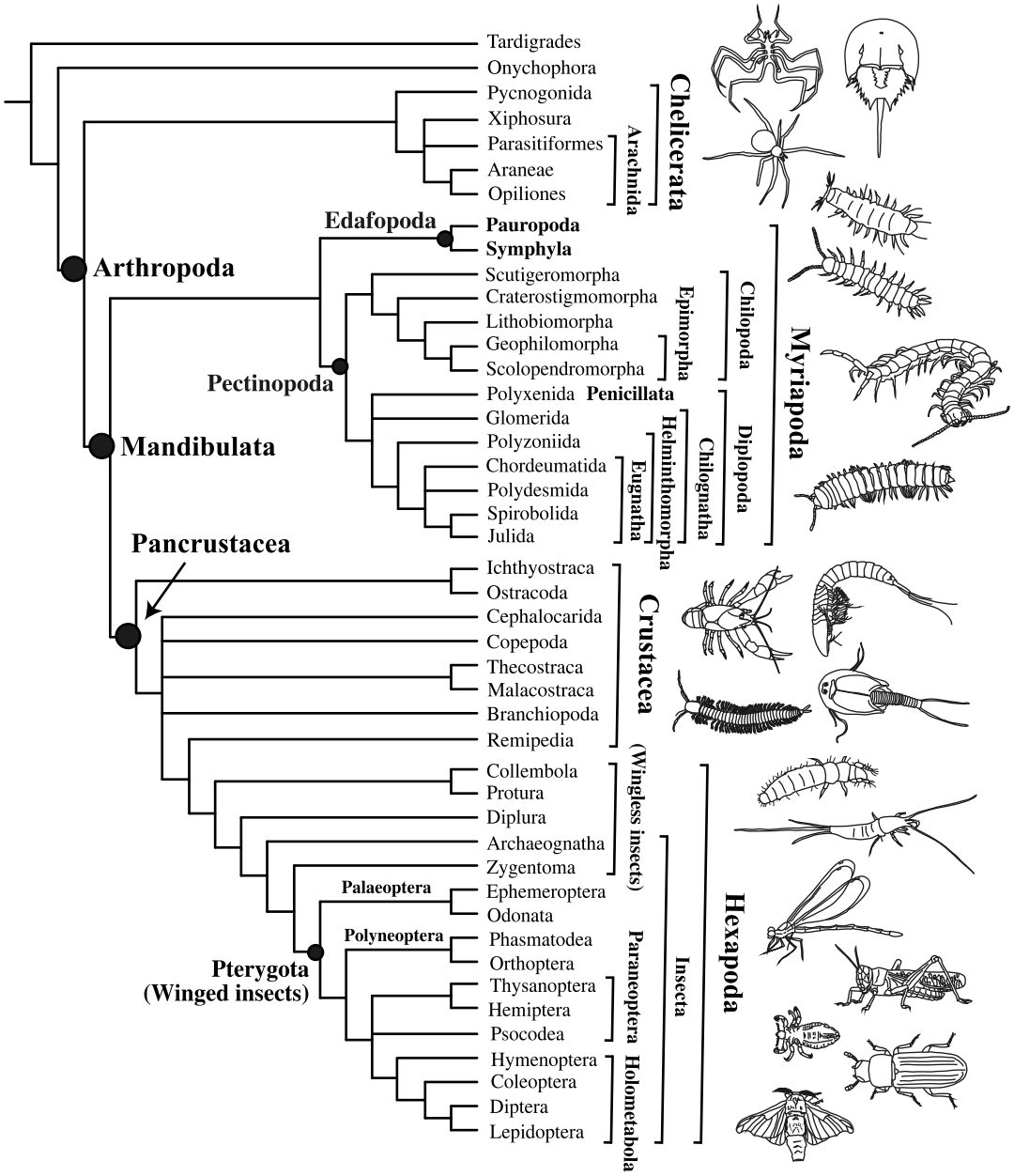

Fig. 5. Arthropod phylogeny strongly supported across all phylogenetic analyses conducted in this study.

maxillary segment, represent traditional views within Myriapoda phylogeny (Pocock 1893a, 1893b; Tiegs 1947; Dohle 1980). In particular, the Progoneata–Dignatha hypothesis has received widespread acceptance as the prevailing model and is also supported by some molecular phylogenetic evidence based on transcriptome sequence data (Fernández et al. 2018) and mitochondrial gene sequences (Zuo et al. 2022). Conversely, the Progoneata–Edafopoda hypothesis (Fig. 1c), which suggests a sister-relationship between Symphyla and Pauropoda, emerged from molecular phylogenetic analyses based on 62 protein-coding genes (Regier et al. 2010), with the term Edafopoda formally introduced by Zwick et al. (2012). This hypothesis was also supported by a recent reanalysis based on the original dataset of Regier et al. (2010) and Noah et al. (2020) and an analysis of complete mitochondrial genomic sequences (Dong et al. 2012a, 2012b). Phylogenetic analyses utilizing three nuclear genes encoding DNA and RNA polymerases suggested that Symphyla may be considered a sister group to the remaining three myriapod classes, with a notable association between the Chilopoda and Diplopoda (Fig. 1d) (Miyazawa et al. 2014). These phylogenetic relationships were corroborated by an analysis of 454 transcriptome sequences, although the analysis lacked

samples of Pauropoda (Rehm et al. 2014). The grouping of Chilopoda and Diplopoda was also strongly supported by several recent studies employing transcriptome sequence data (Szucsich et al. 2020; Wang et al. 2021; Benavides et al. 2023), culminating in the formal designation of this group as Pectinopoda by Benavides et al. (2023) (see Fig. 1e). Furthermore, these studies also support the taxon Edafopoda, i.e. the hypothesized sister-relationship between Symphyla and Pauropoda (Fig. 1e).

The findings of this study provide strong support for the Edafopoda–Pectinopoda hypothesis (Fig. 1e), i.e. the grouping of Chilopoda and Diplopoda (bootstrap support value [BS]: 88% to 100% and posterior probability [PP]: 1.00) and that of Symphyla and Pauropoda (BS: 97% to 100% and PP: 1.00). However, no evidence was obtained in support of other hypotheses shown in Fig. 1, despite various phylogenetic analyses being conducted using different datasets (Figs. 3 and 4 and see also supplementary figs. S1 to S3 and S6 to S8, Supplementary Material online). Taken together with the results of the recent studies mentioned above (Miyazawa et al. 2014; Rehm et al. 2014; Szucsich et al. 2020; Wang et al. 2021; Benavides et al. 2023), we propose that the Edaphopoda–Pectinopoda hypothesis accurately reflects the true phylogenetic relationships among myriapod classes.

On the other hand, little morphological evidence exists to support the Edaphopoda–Pectinopoda hypothesis. Comb lamellae are present on the distal part of the mandibular gnathal edge in millipedes (Diplopoda) and centipedes (Chilopoda), but absent on the mandibles of symphylans (Symphyla) (Richter et al. 2002; Edgecombe et al. 2003). The lamellae observed on the mandibles of centipedes and millipedes are similar in position, structure, and orientation, suggesting a common origin (Richter et al. 2002; Edgecombe et al. 2003). Therefore, the mandibular comb lamellae currently appear to be the morphological evidence supporting the Pectinopoda (Chilopoda + Diplopoda) (Miyazawa et al. 2014; Szucsich et al. 2020; Benavides et al. 2023), even though mandibular comb lamellae in the Pauropoda have not yet been demonstrated. Although the Edafopoda (Pauropoda + Symphyla) was established and named by Zwick et al. (2012) based on molecular phylogenetic analysis (Regier et al. 2010), no morphological evidence was presented to support this taxon.

In contrast, the traditional taxon Dignatha (Pauropoda + Diplopoda) remains strongly supported by numerous morphological traits (see Benavides et al. 2023). A recent study (Szucsich et al. 2020) questions the homology of morphological traits, such as the tracheal pouches and bothriotricha, which are considered to support the Progoneata–Dignatha hypothesis. In addition, they also discuss morphological characters, such as the (ultra-)structure and position of bothriotricha, coxal apodemes on the anterior face of the coxa, and six-segmented legs, all of which could serve as synapomorphies for Edafopoda. However, even if the morphological characters of the trichobothria, apodemes, and leg segmentation are compatible with Edafopoda, as discussed by Benavides et al. (2023), the Dignatha hypothesis (Pauropoda + Diplopoda) remains a strong morphological hypothesis.

Therefore, there seems to be a significant discrepancy between morphology and molecular phylogeny. As we concluded above, if the Edafopoda–Pectinopoda hypothesis reflects the true phylogenetic relationships of myriapods, then the morphological traits supporting Dignatha are either the result of convergent evolution between Pauropoda and Diplopoda, or they reflect the ancestral traits of myriapods that were lost or changed in centipedes and symphylans during their evolution.

Phylogeny of Chilopoda and Diplopoda

Centipedes (chilopods) are a well-known predatory group because of their potent venom, which is secreted from glands on the modified first pair of trunk legs; they are characterized as having one pair of legs per body segment compared to millipedes, which have two pairs of legs per body segment. The class Chilopoda consists of five orders: Scutigeromorpha, Craterostigmomorpha, Lithobiomorpha, Geophilomorpha, and Scolopendromorpha. The present results based on all phylogenetic analyses show a strongly supported interordinal relationship: (Scutigeromorpha, Craterostigmomorpha, Lithobiomorpha, Geophilomorpha, Scolopendromorpha) (Figs. 3 and 4). This relationship is also supported by numerous previous studies based on both morphological (Dohle 1980, 1985; Edgecombe and Giribet 2002; Edgecombe et al. 1999; Edgecombe and Giribet 2004) and molecular data (Edgecombe et al. 1999; Edgecombe and Giribet 2004; Regier et al. 2008, 2010; Murienne et al. 2010; Szucsich et al. 2020; Benavides et al. 2023), lending considerable support to this view. However, the incongruent placements of Craterostigmomorpha based on morphology and phylogenomics (the Phylactometria vs. Amalpighiata controversy) should be acknowledged (Edgecombe and Giribet 2004; Giribet and Edgecombe 2006), and this may suggest that the Lithobiomorpha have undergone a unique evolution in morphology. Our results also strongly support the validity of the taxon Epimorpha (Dohle 1980) as having a sister-relationship between the two orders Scolopendromorpha and Geophilomorpha. These findings suggest that the epimorphic chilopods have a common origin and that their epimorphic mode of postembryonic development is that of synapomorphy (Dohle 1980).

For millipedes (diplopods), the RNA-seq data for eight species representing seven orders were obtained and used for phylogenetic analysis. All of the resulting trees confirmed the monophyly of both the infraclass

Helminthomorpha and the subclass Eugnatha, and the sister-relationship of the two orders, Spirobolida and Julida (Fig. 5). The monophyly of subclass Chilognatha was supported by the trees generated based on Dataset B (Table 1 and Figs. 3 and 4), but the order Glomerida of the subclass Chilognatha was clustered with the order Polyxenida (subclass Penicillata) in the trees generated based on Dataset A (Table 1 and Fig. 3). Also, the phylogenetic positions of the orders Chordeumatida and Polydesmida were switched between Dataset A and Dataset B (Table 1 and Figs. 3 and 4), except for Dataset A4, which generated the same results as Dataset B (supplementary fig. S4, Supplementary Material online). These results suggest that the phylogenetic positions of the three orders Glomerida, Chordeumatida, and Polydesmida appear to remain an open question that requires further clarification with additional datasets and sample species, although the results inferred from Dataset B are more strongly supported by previous studies (Miyazawa et al. 2014; Szucsich et al. 2020; Benavides et al. 2023).

Phylogeny of Pancrustacea

The Pancrustacea includes paraphyletic crustaceans and monophyletic hexapods. Our phylogenetic analyses included 34 species representing all of the pancrustacean classes and orders that are important for understanding the phylogenetic relationships within the Pancrustacea (supplementary table S1, Supplementary Material online). The following phylogenetic relationships of hexapods were strongly supported on all of the trees generated by the present analyses. (i) The Entognatha, including the three wingless orders, Collembola, Protura, and Diplura, are not monophyletic; Collembola and Protura are clustered into one clade, while Diplura is a sister group to Insecta (Figs. 3 to 5). (ii) Among the five orders of wingless insects (Apterygota), the order Zygentoma is the most closely related to, and forms the sister group of, all winged insects (Pterygota). (iii) For the winged insects, the Palaeoptera (Ephemeroptera + Odonata) split off first, followed by the Polyneoptera (Phasmatodea and Orthoptera, *etc*.), and the Paraneoptera is a sister group to the Holometabola (Figs. 3 to 5). These phylogenetic relationships are completely or partially supported by numerous previous studies (Ishiwata et al. 2011; Sasaki et al. 2013; Misof et al. 2014; Chesters 2017; Noah et al. 2020) and seem to be less controversial. Conversely, based on the results of recent molecular phylogenetic studies, the ordinal relationships within the superorder Paraneoptera are highly controversial. The Paraneoptera consists of three hemipteroid insect orders, Psocodea (lice), Thysanoptera (thrips), and Hemiptera (true bugs), and is traditionally considered to be a monophyletic group based on morphological characters (Grimaldi and Engel 2005). Molecular phylogenetic analyses based on three nuclear protein-coding gene sequences suggested that the Psocodea (Psocoptera and Phthiraptera) is relatively closely related to holometabolous insects, implying that the monophyly of the Paraneoptera is not supported (Ishiwata et al. 2011). This finding was confirmed by phylogenomic analyses based on 1,478 (Misof et al. 2014) and 2,395 (Johnson et al. 2018) single-copy protein-coding genes from a comprehensive collection of insects. In contrast, other phylogenomic analyses (Chesters 2017; Lozano-Fernandez et al. 2019a, 2019b; Thomas et al. 2020) combined the three orders of hemipteroid insects into a single clade, supporting the monophyly of Paraneoptera. However, different results were obtained in this study: Dataset A supports Psocodea as a sister group of Holometabola, while Dataset B supports the monophyly of Paraneoptera. In both datasets, differences among samples did not affect the results (Figs. 3 and 4). Thus our results suggest that the phylogenetic relationships among the Paraneoptera are highly dependent upon the sequence datasets. It will be important to determine which datasets reflect the true phylogenetic relationships as a future challenge, but this is currently unknown. Until this issue is resolved, we will not be able to understand the origin of holometabolous insects.

The other important phylogenetic issue related to Pancrustacea is the question of which crustaceans are most closely related to hexapods, as this is key to understand the origin of hexapods. Recent studies have proposed two hypotheses: either the Remipedia alone or both the Remipedia and the Cephalocarida are closely related to the hexapods (Schwentner et al. 2018; Lozano-Fernandez et al. 2019a, 2019b; Noah et al. 2020; Bernot et al. 2023; Yu et al. 2024). The findings of our study strongly support the former hypothesis, namely that remipedes are a sister group to hexapods (Figs. 3 and 4). An examination of this and previous studies reveals that analyses using a larger number of genes and more extensive sampling appear to support the former of the two hypotheses. It is noteworthy that the study by Yu et al. (2024) indicates that the close relationship between Remipedia and the Cephalocarida is due to systematic errors. Compared to hexapods, the phylogenetic relationships of crustaceans were less well resolved in the present study, with the exception of the sister-relationship between Ichthyostraca and Ostracoda and between Thecostraca and Malacostraca. The findings of this study also strongly supported the hypothesis that the Ichthyostraca + Ostracoda clade was the first to diverge from the pancrustaceans (Figs. 3 to 5). Overall, this study showed relatively low consistency for the phylogenetic relationships among crustacean classes (Figs. 3 to 5). However, these results may also suggest that numerous clades of crustaceans underwent rapid radiation in a short period of time.

Materials and Methods

Samples

A total of 64 species representing all arthropod classes were analyzed in this study; three chelicerate classes: Pycnogonida, Merostomata, and Arachnida; four myriapod classes: Chilopoda, Diplopoda, Pauropoda, and Symphyla; six crustacean classes: Ostracoda, Hexanauplia, Malacostraca, Branchiopoda, Cephalocarida, and Remipedia; two hexapod classes: Entognatha and Ectognatha. Of these, 28 species (10 crustaceans, 13 myriapods, and 5 chelicerates), which were collected from the Ryukyu Islands and the Kansai region of Japan, were used for transcriptome sequencing. The transcriptome sequences of other arthropod species and five outgroup species were retrieved from the National Center for Biotechnology Information sequence database (supplementary table S1, Supplementary Material online).

RNA Extraction, Library Preparation, and High-throughput Sequencing

Total RNA was extracted from living samples or samples that had been stored at −80 °C using an ISOGEN kit (Nippon Gene). The total RNA was used to construct an RNA library with TruSeq RNA Library Preparation Kit v2 (Illumina). High-throughput Sequencing (HTS) was performed with MiSeq reagent Kit v3 (300 bp paired-end) on MiSeq (Illumina), except for the four myriapod samples (my118, my119, my125, and my128), which were sequenced on an HiSeq X Ten system (Illumina). Sequence assembly was performed using CLC Genomic Workbench (Filgen) with five word sizes (21, 31, 41, 51, and 61 bp). Similar isoforms within each assembly were eliminated using CD-HIT-EST (version 4.6.1) (Fu et al. 2012), with an 80% similarity cut-off to reduce redundancy. Contigs shorter than 500 bp were discarded. The number of reads and contigs of the transcriptome data are shown in supplementary table S1, Supplementary Material online.

Identification of Orthologous Protein-coding Genes

HaMStR version 13.2.3 (Ebersberger et al. 2009) was used to search the assembled transcriptome data to identify protein sequences that matched two different gene sets referred to as arthropod core orthologues (http://hmmer.org/download.html) (Ebersberger et al. 2009). The first core orthologue set (Coreset A: insecta_hmmer3_2) includes 1,579 single-copy orthologous genes predefined from the genome sequences of *Apis mellifera*, *Tribolium castaneum*, *Bombyx mori*, *Daphnia pulex*, *Ixodes scapularis*, and *Capitella* sp. The second set (Coreset B: arthropoda_hmmer3) consists of 1,013 single-copy orthologous genes derived from the genome sequences of *A. mellifera*, *Drosophila melanogaster*, *B. mori*, *T. castaneum*, *Pediculus humanus*, *Daphnia pulex*, *Caenorhabditis elegans*, and *Pristionchus pacificus*. The number of orthologous genes obtained from the transcriptome data for each species is shown in supplementary table S1, Supplementary Material online.

Dataset Construction and Phylogenetic Analyses

For dataset construction, five sample sets were prepared according to the focus of phylogenetic analysis. The sample set 1 includes all samples, and the sample sets 2 to 5 focus on analyzing the phylogenetic relationships among (i) arthropod subphyla, (ii) myriapod classes, (iii) orders of two myriapod classes (Chilopoda and Diplopoda), and (iv) higher taxa in pancrustaceans (crustaceans + hexapods), respectively (Table 1 and supplementary tables S2 to S11, Supplementary Material online). The rationale employed for using these sample sets was 2-fold. One was to clarify whether the results of phylogenetic analysis differ among the different sample sets. The other was to perform phylogenetic analysis using more appropriate outgroups. The protein sequences of the orthologous genes, identified from the transcriptomes by HaMStR and based on the two coresets (Ebersberger et al. 2009), were aligned using MAFFT L-INS-I (Katoh et al. 2005) for each sample set. The poorly aligned positions and divergent regions were eliminated using Gblocks 0.91b (Castresana 2000) with default parameters. In addition, genes shorter than 100 aa were discarded using Seqkit (https://bioinf.shenwei.me/seqkit/) (Shen et al. 2016), and the remaining genes containing at least 100 aa were concatenated to form a super-matrix for each dataset. The Perl scripts for multiMafft, multiGblocks, and sequence concatenation were downloaded from Inoue's website and modified (http://www.fish-evol.org/MAFFT.html and http://www.fish-evol.org/Perl_JI.html). Since we found that the super-matrix still had some poorly aligned positions and divergent regions, we reperformed the steps for removing ambiguous positions using Gblocks (Castresana 2000) on Seaview version 4.7 (Gouy et al. 2010) using the following parameters: (i) allow smaller final blocks, (ii) allow gap positions within the final blocks, and (iii) allow less strict flanking positions. The workflow of dataset construction is shown in Fig. 2. Information regarding the data used for the final super-matrices is shown in Table 1 and supplementary tables S2 to S11, Supplementary Material online.

The FASTA format files of the super-matrixes compiled for each dataset, as described above, were converted to PHYLIP format using Seaview (Gouy et al. 2010), and to NEXUS format using MEGA X (Kumar et al. 2018). These files were then subjected to phylogenetic analyses with RAxML (version 8.0.0; Stamatakis 2014) and MrBayes (version 3.2.7a; Ronquist et al. 2012), respectively. All free aa

model parameters were automatically estimated by the RAxML (Stamatakis 2014), and LG was identified as the best-scoring aa model for all datasets. Therefore, RAxML (Stamatakis 2014) analysis was carried out using a LG + GAMMA model, with bootstrapping performed using 200 replicates. Bayesian inference (BI) analysis was performed using the MrBayes (Ronquist et al. 2012) under the LG + GAMMA model with two separate runs, each comprising four chains for 200,000 generations. The trees were sampled every 1,000 generations and the convergence of runs was assessed with Tracer v1.7.1 (Rambaut et al. 2018), with the first 25% of trees discarded as burn-in, by default. Due to limitations in computing power, BI analysis was not performed for the two datasets A1 and B1, which included sequence data from all samples. Seaview (Gouy et al. 2010) was used to output the trees (in Newick file format) inferred by RAxML (Stamatakis 2014) and MrBayes (Ronquist et al. 2012).

Supplementary Material

Supplementary material is available at *Genome Biology and Evolution* online.

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Author Contributions

Z.-H.S. designed the study, performed phylogenetic analyses, and wrote the paper with input from the other authors. Z.-H.S., H.M., and A.S. collected samples. H.M. and A.S. performed NGS sequencing. A.S. and Z.-H.S. prepared datasets for phylogenetic analyses. K.O. contributed to writing some scripts for dataset preparation. All authors contributed to discussing results.

Data Availability

The transcriptome sequence data have been deposited in DDBJ/EMBL/GenBank under the following accession numbers: DRR345786–DRR345813. The super-matrix datasets of amino acid sequences and tree files can be obtained from the DRYAD database (https://doi.org/10.5061/dryad.p8cz8w9th).

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