

# Decisive Data Sets in Phylogenomics: Lessons from Studies on the Phylogenetic Relationships of Primarily Wingless Insects

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## Abstract

Phylogenetic relationships of the primarily wingless insects are still considered unresolved. Even the most comprehensive phylogenomic studies that addressed this question did not yield congruent results. To get a grip on these problems, we here analyzed the sources of incongruence in these phylogenomic studies by using an extended transcriptome data set. Our analyses showed that unevenly distributed missing data can be severely misleading by inflating node support despite the absence of phylogenetic signal. In consequence, only decisive data sets should be used which exclusively comprise data blocks containing all taxa whose relationships are addressed. Additionally, we used Four-cluster Likelihood Mapping (FcLM) to measure the degree of congruence among genes of a data set, as a measure of support alternative to bootstrap. FcLM showed incongruent signal among genes, which in our case is correlated neither with functional class assignment of these genes nor with model misspecification due to unpartitioned analyses. The herein analyzed data set is the currently largest data set covering primarily wingless insects, but failed to elucidate their interordinal phylogenetic relationships. Although this is unsatisfying from a phylogenetic perspective, we try to show that the analyses of structure and signal within phylogenomic data can protect us from biased phylogenetic inferences due to analytical artifacts.

**Key words:** phylogenomics, ESTs, likelihood quartet mapping, conflicting hypotheses, Entognatha, Nonoculata, Ellipura, Protura, Diplura, Collembola, missing data.

## Introduction

Despite enormous efforts to resolve the tree of life, several deep nodes are still considered unresolved. A good example for such problems are the unresolved phylogenetic relationships of primarily wingless insects.

Most phylogenetic studies including multigene and phylogenomic analyses have recovered the monophyly of Hexapoda, the insect clade in a broad taxonomic sense (Regier et al. 2008, 2010; von Reumont et al. 2009, 2012; Meusemann et al. 2010; Trautwein et al. 2012). Furthermore, the monophyly of Ectognatha, which comprises

insects in a strict taxonomic sense, namely jumping bristletails, silverfishes and firebrats, and winged insects, is well supported (reviewed in Grimaldi 2010; Trautwein et al. 2012). By contrast, phylogenetic relationships among the entognathous primarily wingless insects, the Protura (coneheads), Collembola (springtails), and Diplura (two-pronged bristletails), are unclear. Many authors consider these entognathous insects as being monophyletic, considering entognathy in which mouth parts are concealed in gnathal pouches (first discussed in detail by Hennig 1953) to have evolved in the last common ancestor of the three groups.

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Within Entognatha, either a clade uniting Protura and Collembola, referred to as Ellipura (Börner 1910), or a clade uniting Protura and Diplura, referred to as Nonoculata (Luan et al. 2005), has been proposed (Ellipura [Hennig 1953; Kristensen 1981, 1997; Shao et al. 1999; Bitsch and Bitsch 2000, 2004; Carapelli et al. 2000; Zhang et al. 2001]; Nonoculata [Giribet and Wheeler 2001; Giribet et al. 2004; Luan et al. 2005; Kjer et al. 2006; Mallatt and Giribet 2006; Misof et al. 2007; Dell'Ampio et al. 2009; von Reumont et al. 2009; Mallatt et al. 2010]). Other authors consider a paraphyly of Entognatha to be more likely, with Diplura as closest relatives to Ectognatha. Possible arguments for this hypothesis include the evolutionary origin of paired pretarsal claws and paired cerci (Kukalová-Peck 1987; Koch 1997; Beutel and Gorb 2006), the ultrastructure of the sperm (Dallai et al. 2011), and the differentiation process of the embryonic amnion (Machida 2006) in the last common ancestor of Diplura and Ectognatha.

Meusemann et al. (2010) and von Reumont et al. (2012) published the most relevant data sets and analyses covering the phylogenetic relationships among primarily wingless insects by including expressed sequence tag (EST) data of representatives of Protura, Collembola, and Diplura. Although both studies recovered the monophyly of Entognatha, Meusemann et al. found strong evidence for Protura and Diplura as closest relatives (i.e., Nonoculata) and von Reumont et al. for Protura and Collembola as closest relatives (i.e., Ellipura). These incongruent results are puzzling because taxon sampling of the primarily wingless insects is comparable in both studies, as well as the strategies used for orthology assignment, alignment masking, matrix optimization, and tree inference.

These special circumstances put us into the exceptionally favorable position to analyze possible sources of incongruence among these two large phylogenomic data sets. Most phylogenomic studies are based on concatenated supermatrices with low gene data coverage. Focusing on relationships among specific groups, many data blocks within such supermatrices therefore may not contain data for all taxa under consideration. Consequently, our starting hypothesis was that extensive missing data may mislead proper tree reconstruction. To tackle this problem, we complement the publicly available EST data of primarily wingless insects with additional EST data from representatives of Japygidae (Diplura) and Zygentoma (silverfishes and firebrats). We took particular care to concatenate a data set that contains only gene data blocks for which entognathous hexapods and outgroups had gene data coverage. We call such a data set in the following a decisive data set. Note that the term decisiveness has been used before in the context of phylogenomic data sets (Steel and Sanderson 2010; Sanderson et al. 2010), albeit based on a distinct criterion. The concatenated data set is the largest known data set covering primarily wingless insects. It was this data set that allowed us to analyze the effect of the observed uneven distribution of missing data on the extent of bootstrap support (BS). Complementary to the application of BS measures, we applied a Four-cluster Likelihood Mapping (FCLM) approach (Strimmer and von Haeseler 1997), which

has been shown to be effective in disentangling signal among four groups of species. The application of bootstrapping and FCLM helped to assess the effect of the uneven distribution of missing data in indecisive data sets. Complementary to the previously mentioned analyses, we addressed the problem of incongruent signal among genes in a multigene data set by comparing tree reconstructions based on the entire decisive data set with tree reconstructions based on subsets of genes that support incongruent hypotheses. Altogether, our approach provides potential explanations for contradictory results among phylogenomic studies by pointing out underestimated sources of error and incongruence.

## Results

### Orthology Assignment, Alignment, and Alignment Masking

Using the reference set of 1,886 1:1 orthologous genes (OGs), we identified between 52 and 682 putative 1:1 orthologous transcripts in the transcriptome assemblies of primarily wingless hexapods (table 1) and up to 1,886 for all taxa (supplementary table S1, Supplementary Material online). We excluded 20 OGs that were present in the five reference species but absent from all other species from subsequent analyses. After alignment masking (i.e., the exclusion of multiple sequence alignment sections in which sequence similarity cannot be distinguished from random similarity of sequences), the concatenated superalignment was composed of 73 taxa with a total alignment length of 881,235 amino acid sites, partitioned into 1,866 genes (supplementary fig. S1; for gene annotations, see supplementary table S2, Supplementary Material online).

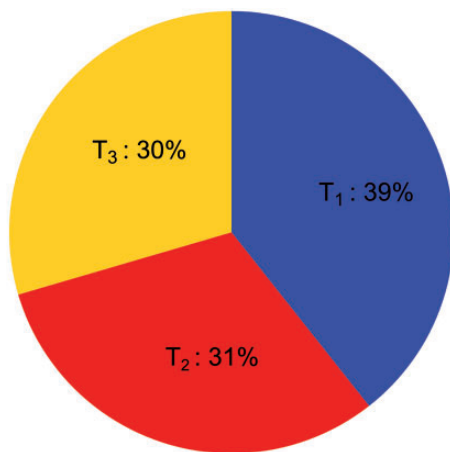
### Relationships among Entognathous Hexapod Lineages

The data set *M\_Ento*, which is decisive for addressing relationships among the three entognathous groups, Protura, Collembola, and Diplura (73 taxa, 117 genes, 32,883 aligned aa sites), moderately supported a clade Protura + Diplura (Nonoculata) (fig. 1). This is compatible with the results of the FCLM approach (topology  $T_1$  favored, fig. 2). Tree reconstruction supported Collembola as closest relatives to a clade comprising Nonoculata and Ectognatha. The clade Nonoculata + Ectognatha received moderate support (fig. 1; supplementary fig. S2, Supplementary Material online).

Our tree reconstructions based on a selected optimal subset (SOS) extracted from a complete data matrix by optimizing information content and data saturation in iterative steps of gene and/or taxon exclusion (see MARE manual; Meusemann et al. 2010; Meyer and Misof 2010) (62 taxa, 253 genes, alignment length 55,429 aa positions) yielded monophyletic Entognatha with moderate support and Nonoculata with low support (fig. 3a and table 2; supplementary fig. S3a, Supplementary Material online). It should be kept in mind that this SOS is indecisive for addressing the relationships of Entognatha, with only one-third of all genes (79) of this data set being covered by all three entognathous groups (supplementary table S3, Supplementary Material online). The tree based on the data set  $SOS_{60}$ , in which



(Protura + Collembola) – (Diplura + remaining taxa) in 35 genes (11,789 aligned aa positions) (data set *M\_Elli*, derived from Ellipura), and a predominant signal for topology  $T_3$  (Diplura + Collembola) – (Protura + remaining taxa) in 31 genes (8,546 aligned aa positions) (data set *M\_DiCo*) (fig. 4a and b). Tree inferences from data sets *M\_Nono*, *M\_Elli*, and *M\_DiCo* (rogue taxa pruned, see Materials and Methods section) yielded maximal BS support for Nonoculata, Ellipura, and Diplura + Collembola, respectively (table 2; supplementary fig. S4, Supplementary Material online). However, although tree reconstruction of our data subsets *M\_Nono*, *M\_Elli*, and *M\_DiCo* showed maximal BS support for incongruent topologies among the entognathous insect orders, the results from the FcLM approach indicated that signal for alternative topologies was present in all data sets (fig. 4a and b; supplementary table S4, Supplementary Material



**Fig. 2.** Results of the FcLM for all OGs in data set *M\_Ento*. The chart shows the proportion of quartets (summed up for 117 OGs) that show predominant support for  $T_1$  ([Protura + Diplura] – [Collembola + remaining taxa], Nonoculata hypothesis, blue),  $T_2$  ([Protura + Collembola] – [Diplura + remaining taxa], Ellipura hypothesis, red), and  $T_3$  ([Diplura + Collembola] – [Protura + remaining taxa], yellow), see fig. 5. Quartets mapping in remaining Voronoi cells (gray) and  $T^*$  (fig. 5) were not considered.

online), which is not reflected by the trees. To identify possible reasons for incongruent signal among genes, we assessed the correlation between functional classes of genes and the different phylogenetic hypotheses that are supported by the data subsets. We found no correlation (supplementary material [section 4], table S5 and fig. S5, Supplementary Material online). Additionally, we tested whether model misspecification can explain the observed incongruence among genes and analyzed the data set *M\_Ento* and data subsets *M\_Nono*, *M\_Elli*, and *M\_DiCo* using partitioned phylogenetic analyses (Minh et al. 2013) with the best model selected for each gene (partition) separately (supplementary material [section 5], table S6, and figs. S6–S9, Supplementary Material online). With respect to the phylogenetic relationships addressed in our study, resulting topologies did not differ from unpartitioned analyses, and BS only differed to a minor degree (table 2).

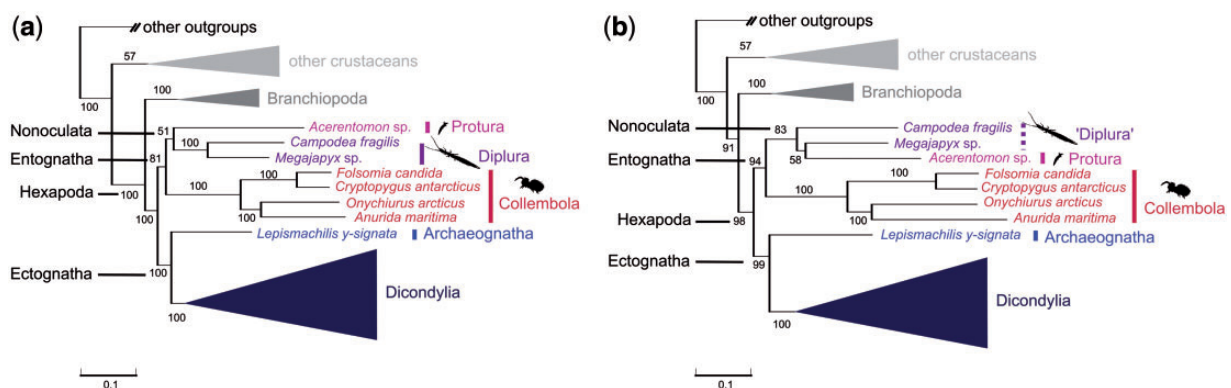
## Discussion

### The Importance of Data Set Decisiveness

Incongruences in proposed relationships among Protura, Collembola, and Diplura in the studies of Meusemann et al. (2010) and von Reumont et al. (2012), which both supported monophyly of Entognatha, motivated us to look for new approaches to uncover and analyze possible sources of incongruent signal in phylogenomic data sets.

Both SOS data sets in Meusemann et al. (2010) and von Reumont et al. (2012) were compiled with MARE (Meyer and Misof 2010) and were intended to address pancrustacean and arthropod relationships. Both data sets showed only low decisiveness for addressing the relationships of the three entognathous lineages: only 28 out of 128 genes in Meusemann et al. (2010) and 22 out of 316 genes in von Reumont et al. (2012) contained representatives of Protura, Diplura, and Collembola.

Despite low gene data coverage in both studies, the monophyly of Entognatha received high BS. By contrast, our decisive data set for addressing the relationships among these three insect orders lacks clear support for Entognatha



**Fig. 3.** Simplified phylogenetic trees of data sets SOS (a) and  $SOS_0$  (b). Best ML tree (RAXML v.7.2.8, PROTCAT, LG + GAMMA) (a) based on 253 OGs, 79 of which are covered by Protura, Diplura, and Collembola (SOS) and (b) based on 174 OGs, none of which are covered by Protura, Diplura, and Collembola ( $SOS_0$ ). BS is derived from 1,000 bootstrap replicates. Trees were rooted with *Capitella* sp. For the full trees, see supplementary figure S3a and S3b, Supplementary Material online.

**Table 2.** BS (%) for Selected Clades in Tree Reconstructions with Various Data Sets.

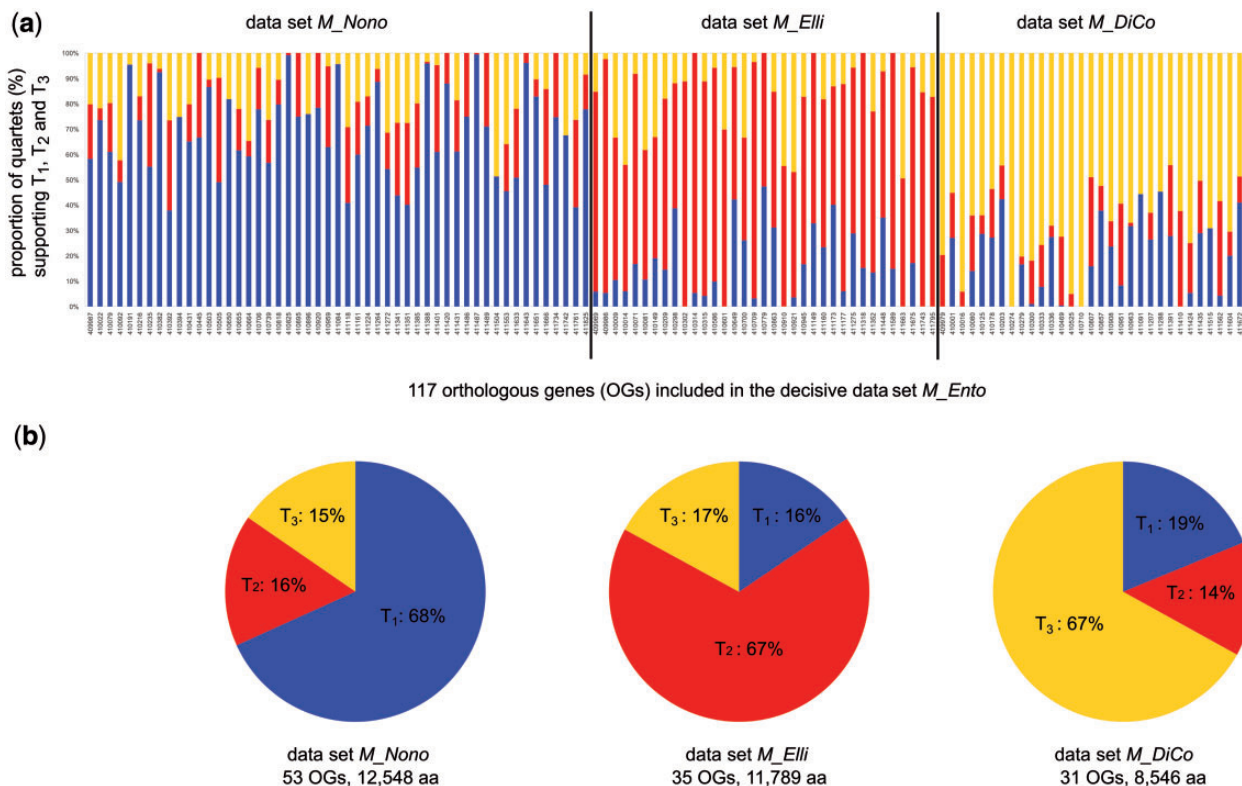
Clade	Data Set					Data Subset of <i>M_Ento</i>		
	<i>M_Ento</i>	SOS	SOS <sub>69</sub>	Meusemann et al. (2010)	von Reumont et al. (2012)	<i>M_Nono</i>	<i>M_Elli</i>	<i>M_DiCo</i>
Hexapoda	100 (100)	100	98	100	99	72 (100)	100 (100)	100 (100)
Diplura	100 (100)	100	— <sup>a</sup>	N.A.	N.A.	100 (100)	100 (100)	100 (100)
Collembola	100 (100)	100	100	100	100	100 (100)	100 (100)	100 (100)
(Protura, Diplura) <sup>b</sup>	91 (96)	51	83 <sup>a</sup>	100	—	100 (100)	— (—)	— (—)
(Protura, Collembola) <sup>c</sup>	— (—)	—	—	—	98	— (—)	100 (100)	— (—)
(Diplura, Collembola)	— (—)	—	—	—	—	— (—)	— (—)	99 (100)
Entognatha	— (—)	81	94	86	98	— (—)	— (—)	— (—)
((Protura, Diplura), Ectognatha)	80 (96)	—	—	—	—	98 (100)	— (—)	— (—)
((Collembola, Diplura), Ectognatha)	— (—)	—	—	—	—	— (—)	— (—)	60 (83)
(Diplura, Ectognatha)	— (—)	—	—	—	—	— (—)	66 (100)	— (—)
Ectognatha	100 (100)	100	99	100	100	100 (100)	100 (100)	95 (84)

NOTE.—BS was assessed with RAxML from 1,000 bootstrap replicates (see Materials and Method). BS printed in brackets was assessed from partitioned ML analyses of data sets *M\_Ento*, and its subsets using the Uboot algorithm of IQ-TREE with 5,000 bootstrap replicates (supplementary material [section 5], Supplementary Material online). *M\_Ento* is the decisive data set in which all OGs are covered by Protura, Diplura, and Collembola; SOS, SOS<sub>69</sub>, and the data sets from Meusemann et al. (2010; data set SOS, ML tree) and von Reumont et al. (2012; data set SOS, ML tree Set 1<sub>red</sub>) are indecisive to address the relationships of entognathous hexapod orders. *M\_Nono*, *M\_Elli*, and *M\_DiCo* are subsets of *M\_Ento* with predominant signal for different topologies and point out conflict of signal among genes.

<sup>a</sup>Diplurans are paraphyletic: *Campodea* + (*Acerentomon*, *Megajapyx*).

<sup>b</sup>Nonoculata hypothesis.

<sup>c</sup>Ellipura hypothesis.



**FIG. 4.** Detailed results of the FcLM Mapping for all OGs included in data set *M\_Ento* and data subsets *M\_Nono*, *M\_Elli*, *M\_DiCo*. (a) Histogram of FcLM results. Each bar refers to an OG (for OG-IDs, see supplementary table S2, Supplementary Material online). Y axis: amount of quartets (in %), that predominantly support T<sub>1</sub> ([Protura + Diplura] – [Collembola + remaining taxa], blue), T<sub>2</sub> ([Protura + Collembola] – [Diplura + remaining taxa], red), and T<sub>3</sub> ([Diplura + Collembola] – [Protura + remaining taxa], yellow), quartets that show ambiguous support are not considered (fig. 5). OGs with predominant support for T<sub>1</sub> are classified into data set *M\_Nono* (51 genes, 12,548 aligned aa positions); OGs with predominant support for T<sub>2</sub> are classified into data set *M\_Elli*; (35 genes, 11,789 aligned aa positions); OGs with predominant support for T<sub>3</sub> are classified into data set *M\_DiCo* (31 genes, 8,546 aligned aa positions). (b) FcLM results for data set *M\_Nono* (left), *M\_Elli* (middle), and *M\_DiCo* (right). Each chart shows the proportion of quartets (summed up for the OGs included in the data sets) that show predominant support for T<sub>1</sub>, T<sub>2</sub>, and T<sub>3</sub> (see above and fig. 5). Quartets that show ambiguous support (fig. 1) are not considered.

(fig. 1). This puzzling result might be explained by the presence of an uneven distribution of missing data. We gained indirect evidence for this hypothesis with the analyses of the worst case data set  $SOS_{\omega}$ . This data set is maximally indecisive for testing the monophyly of Entognatha, that is, none of the included genes were common to all three entognathous insect groups. Any inferred support for this clade in the  $SOS_{\omega}$  analysis can be considered an artifact. Remarkably, bootstrapping delivered high, clearly artificial support for monophyletic Entognatha in the  $SOS_{\omega}$  tree (fig. 3b).

We conclude from this indirect evidence that the support for Entognatha in Meusemann et al. (2010), von Reumont et al. (2012) and in our data set indecisive concerning this question (fig. 3a) probably results from an artificial signal due to uneven distribution of missing data (Philippe et al. 2011) among Protura, Diplura, and Collembola.

Based on the analyses of the decisive and indecisive data sets, we reject the hypothesis that missing data are unproblematic as long as many characters have been sampled overall (Wiens 2006). Missing data can be misleading as shown by the worst case  $SOS_{\omega}$  data set analysis, in which relationships received high BS although the data set was maximally indecisive. Therefore, we strongly advocate the exclusive use of decisive data sets in phylogenomic studies.

### Incongruent Signal between Genes in a Multigene Data Set

Even decisive data sets can contain incongruent signal (Degnan and Rosenberg 2009; Knowles 2009; Philippe et al. 2011). Using FcLM, we identified groups of genes that support different relationships of Protura, Collembola, and Diplura in the decisive data set  $M\_Ento$  (fig. 4a and b). Additionally, we assessed conflict within the data with split analyses relying on NeighborNetworks (supplementary material [section 6] and figs. S10–S13, Supplementary Material online). This analysis corroborates the results of FcLM that all analyzed data sets did contain incongruent signal. Additional to the problem of indecisiveness discussed earlier, this incongruent signal among genes may partly be responsible for the contradictory results of Meusemann et al. (2010) and von Reumont et al. (2012). However, incongruent signal among genes is difficult to address and rectify. We analyzed two potential sources of conflict and can conclude that both can be excluded. First, we tested for homoplasy due to analogous selection regimes in functional complexes but found no correlation between predicted gene function and phylogenetic signal (supplementary material [section 4], fig. S5, and table S5, Supplementary Material online). Second, we were able to indirectly exclude model misspecifications as sources of incongruent signal because unpartitioned and partitioned maximum likelihood (ML) analyses yielded topologically congruent results and almost identical BS (table 2; supplementary material [section 5], table S6, and figs. S6–S9, Supplementary Material online). With respect to the FcLM, it may well be that this likelihood mapping approach selects sets of genes with congruent substitution processes. A possible solution, but certainly not a fully satisfying one, would be to increase the number of genes to minimize noise and confounding signal.

## Relationships of Protura, Collembola, and Diplura

### Monophyly of Entognatha

The monophyly of Entognatha has never been maximally supported and this has not changed in our analyses (table 2). Studies encompassing representatives of Protura, Collembola, and Diplura are limited to only a few analyses (Colgan et al. 1998; Carapelli et al. 2000; Edgecombe et al. 2000; Giribet et al. 2001, 2005). Monophyletic Entognatha were recovered in all recent studies based on nuclear rRNA genes (Gao et al. 2008; Dell'Ampio et al. 2009; von Reumont et al. 2009; Mallatt et al. 2010). However, BS was low, which was either explained by character choice (Dell'Ampio et al. 2009) or the influence of nonstationary processes across taxa (von Reumont et al. 2009). From the morphological point of view, most apomorphies suggesting the monophyly of Entognatha represent reductions (malpighian papillae vs. tubules; reduction to loss of compound eyes). The only exception is the evolution of mouthparts that are concealed in gnathal pouches (Beutel and Gorb 2006). Diplura as closest relatives to Entognatha is the only relation that contradicts monophyletic Entognatha, and for which morphological evidence has been published (Kukalová-Peck 1991; Koch 1997; Beutel and Gorb 2006; Dallai et al. 2011). In general, morphological support for any clade encompassing more than one of the entognathous lineages Protura, Diplura, and Collembola is weak, largely because character polarization is problematic. This is due to the lack of applicability of characters and/or missing comparative studies in the crustacean groups that are discussed to be most closely related to Hexapoda (Szucsich and Pass 2008).

### Ellipura versus Nonoculata

Molecular analyses mostly support Nonoculata (Protura + Diplura) (Giribet et al. 2004; Luan et al. 2005; Kjer et al. 2006; Mallatt and Giribet 2006; Misof et al. 2007; Dell'Ampio et al. 2009; von Reumont et al. 2009; Mallatt et al. 2010; see Dell'Ampio et al. 2011 for a review) while most morphologists merge Protura and Collembola into Ellipura (Börner 1910; Hennig 1953; Kristensen 1981, 1997; Kukalová-Peck 1987; Bitsch and Bitsch 2000, 2004; Beutel and Gorb 2006). Molecular evidence for Ellipura is weak and limited to three mitochondrial single-gene analyses (Shao et al. 1999; Carapelli et al. 2000; Zhang et al. 2001), and morphological support for Nonoculata is nearly missing (Szucsich and Pass 2008). These controversies call for phylogenomic approaches.

The majority of the 117 genes that compose the decisive data set  $M\_Ento$  contain predominant signal for Nonoculata (fig. 4a). Also, the FcLM analysis of  $M\_Ento$  (fig. 2) and the phylogenetic tree of  $M\_Ento$  (fig. 1) yielded monophyletic Nonoculata, albeit not being well supported. In summary, Nonoculata is slightly favored over Ellipura in our study, but the question of the phylogenetic relationships of the three entognathous hexapod orders remains unsettled.

## Conclusions

Clades may be incorrect, even if receiving high BS support (e.g., monophyly of Entognatha in Meusemann et al. [2010], von Reumont et al. [2012], and in data sets  $SOS$  and  $SOS_{\omega}$  of

this study). This is a trivial conclusion and different reasons are mentioned in the literature (Lehtonen 2011, Simmons and Freudenstein 2011). We show that an uneven distribution of missing data (i.e., the use of indecisive data sets) can lead to strongly supported, yet incorrect, clades. To avoid misleading phylogenetic conclusions from seemingly robust trees based on phylogenomic data sets, we advise 1) using only data sets that are decisive for the phylogenetic question of interest, 2) including an alternative measure of support (Salichos and Rokas 2013); our method of choice was the FcLM approach, and 3) analyzing and documenting the inferred incongruence of signal between genes.

In our decisive data set, we found strong incongruence among genes that is neither correlated with functional classes of genes nor with model misspecifications in unpartitioned analyses. Based upon these notes of caution, we found no signal for the monophyly of Entognatha, and we found no strong signal for Ellipura or Nonoculata despite extending our data set with additional data from key taxa. In other words, the phylogeny and evolution of early hexapods remains enigmatic. Despite this, we show that there are valuable lessons to be learned from the analyses of phylogenomic data of primarily wingless insects, particularly in terms of incongruence among genes and data decisiveness.

## Materials and Methods

### Taxon Sampling and New Transcriptome Data

Our taxon sampling included 73 species: 46 hexapods, and, as outgroup species, 25 crustaceans, the chelicerate *Ixodes scapularis*, and the polychaete worm *Capitella* sp., both present in the reference set of taxa used for orthology assignment (discussed later). Transcriptome assemblies of 71 species were obtained from the Deep Metazoan Phylogeny database (<http://www.deep-phylogeny.org/>, last accessed November 4, 2013). We only used species for which more than 1,000 contigs were available (status: December 2011), with two exceptions: the springtail *Orchesella cincta* (Collembola, Entomobryidae, 754 contigs) and the silverfish *Tricholepisma aurea* (Zygentoma, Lepismatidae, 344 contigs), the only publicly available zygentoman transcriptome assembly (supplementary table S1, Supplementary Material online).

We generated new transcriptome data for *Megajapyx* sp. (Diplura, Japygidae) and the firebrat *Thermobia domestica* (Packard 1837) (Zygentoma, Lepismatidae) (table 1). Extraction of RNA, complementary deoxyribonucleic acid (cDNA) library construction, library normalization, and 454 pyrosequencing of ~1,000,000 ESTs per species using the GS-FLX Titanium System, ROCHE were carried out at the Max Planck Institute for Molecular Genetics (MPIMG), Berlin, Germany. Vector clipping, trimming, and soft masking of raw reads and assembly into contigs was conducted at the Center for Integrative Bioinformatics (CIBIV), Vienna, Austria. Steps at the MPIMG and the CIBIV were done as described in von Reumont et al. (2012) and Simon et al. (2012), for details see supplementary material (section 1; Supplementary Material online). Raw sequence reads were deposited at the National Center for Biotechnology Information (NCBI),

Sequence Read Archive (accession numbers *Megajapyx* sp.: SRR400673; *T. domestica*: SRR400672). Transcriptome assemblies of *Megajapyx* sp. (accession numbers JT047774–JT094274) and *T. domestica* (accession numbers T494145–JT533227) were deposited at the Transcriptome Shotgun Assembly (TSA) Database, NCBI Bioproject ID PRJNA81579 and PRJNA81581 (<http://www.ncbi.nlm.nih.gov/bioproject>, last accessed November 4, 2013). For submission, we excluded contigs shorter than 200 bp, according to the submission guidelines; the full transcriptome assemblies are available at [http://zfmk.de/bioinformatics/Full\\_Transcriptome\\_Assemblies.zip](http://zfmk.de/bioinformatics/Full_Transcriptome_Assemblies.zip) (last accessed November 4, 2013).

### Orthology Assignment

To identify 1:1 OGs in our transcriptome assemblies, we used the Hidden Markov Model based Search for Orthologs using Reciprocity (HaMStR) pipeline (Ebersberger et al. 2009; <http://www.deep-phylogeny.org/hamstr/>, last accessed November 4, 2013), version 4. As reference set for clusters of OGs, we used a set of 1,886 1:1 OGs (represented by amino acid sequences) based on five reference species (supplementary material [section 2] and table S2, Supplementary Material online). We defined orthology being present if bi-directional best hits were found between our transcript sequences and the reference species *Daphnia pulex*, *Ixodes scapularis*, *Apis mellifera*, and *Capitella* sp.

### Alignment, Alignment Masking, and Concatenation

We aligned amino acid sequences using MAFFT L-INS-i (Katoh and Toh 2008) v.6.850 for each gene separately. Afterwards, randomly similar aligned sections were identified with a modified version of ALIScore (Misof B and Misof K 2009; Kück et al. 2010; Meusemann et al. 2010; for modifications, see Meusemann et al. 2010) using the following options: default sliding window size; -r: maximum number of pairwise sequence comparisons; -e: special scoring for gappy amino acid data. Identified randomly similar aligned sections were masked with ALICUT v.2.0 (Kück 2009; [www.utilities.zfmk.de](http://www.utilities.zfmk.de), last accessed November 4, 2013). Masked alignments were concatenated into supermatrices with FASconCAT v.1.0 (Kück and Meusemann 2010).

### Design of Decisive and Indecisive Data Sets

We extracted all genes from the supermatrix that contain at least one representative of each 1) Protura, 2) Diplura, 3) Collembola, and 4) remaining species to generate a decisive data set among entognathous lineages. The resulting data set is called *M\_Ento*.

We generated two additional data subsets from the original supermatrix: 1) A so-called selected optimal subset (SOS), generated with MARE v.0.1.2-rc (Meyer and Misof 2010; <http://mare.zfmk.de>, last accessed November 4, 2013), applying taxon weighting -t 1.5. This approach is analogous to Meusemann et al. (2010) and von Reumont et al. (2012). 2) From this SOS data set, we compiled a data set called SOS<sub>∞</sub> by removing all genes that were covered by all three entognathous lineages to receive a maximally indecisive

“worst case” data set in which each gene contained maximally two entognathous lineages.

#### Four-Cluster Likelihood Mapping

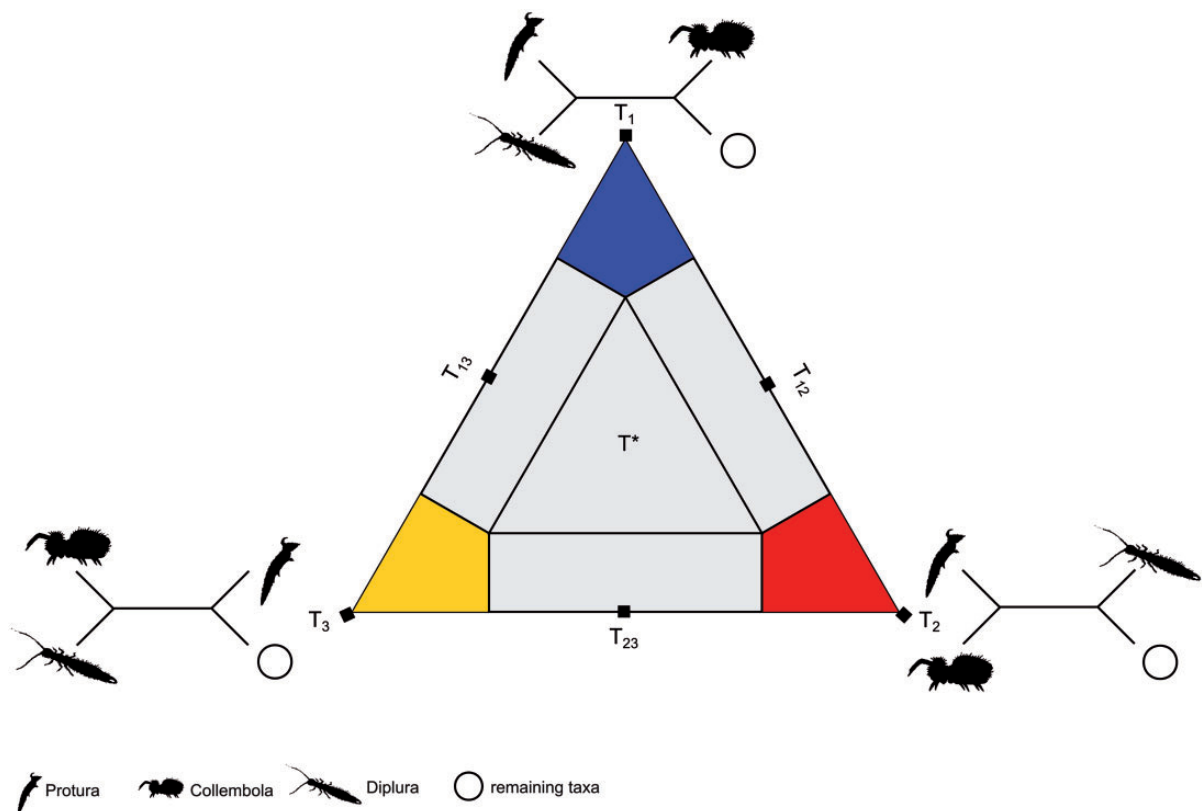
Additional to tree reconstruction with BS, we applied the FcLM approach using the *M\_Ento* data set (Strimmer and von Haeseler 1997). We binned sequenced species into four clusters: 1) Protura (1 species), 2) Diplura (2 species), 3) Collembola (5 species), and 4) remaining species (65 species) (supplementary table S1, Supplementary Material online). Next, we 1) estimated the tree-likeness of each gene, that is the amount of quartets that showed support for one out of the three possible topologies and 2) evaluated which of the three possible topologies was supported by the majority of those quartets (predominant support):  $T_1$  (Protura + Diplura) and (Collembola + remaining taxa),  $T_2$  (Protura + Collembola), and (Diplura + remaining taxa), or  $T_3$  (Diplura + Collembola) and (Protura + remaining taxa) (fig. 5). The competing hypotheses of Meusemann et al. (2010) and von Reumont et al. (2012) are represented by either  $T_1$  (Nonoculata hypothesis) or  $T_2$  (Ellipura hypothesis); the third topology  $T_3$  does not represent a currently debated hypothesis. FcLM was conducted using TREE-PUZZLE v.5.2 (Schmidt et al. 2002; <http://www.tree-puzzle.de>, last accessed November 4, 2013), applying the BLOSUM62 substitution

matrix (Henikoff S and Henikoff JG 1992) as the BLOSUM62 substitution matrix is implemented in the software MARE (Meyer and Misof, 2010; <http://mare.zfmk.de>, last accessed November 4, 2013).

For each gene in the data set *M\_Ento*, we calculated the proportions of quartets that predominantly supported either topology  $T_1$ ,  $T_2$ , or  $T_3$ . According to the topology that was supported by the majority of quartets, we classified each gene into one of three groups, supporting Nonoculata, Ellipura, or Diplura + Collembola (fig. 5 and supplementary table S4, Supplementary Material online). Quartets for which the support remained ambiguous ( $T_{12}$ ,  $T_{23}$ ,  $T_{13}$ , and  $T^*$ ; fig. 5) were not used for classification (see supplementary fig. S14 [Supplementary Material online] for the results with all quartets). All classified genes (supplementary table S4, Supplementary Material online) were subsequently concatenated into three submatrices called *M\_Nono* (genes supporting Nonoculata), *M\_Elli* (genes supporting Ellipura), and *M\_DiCo* (genes supporting Diplura + Collembola).

#### Phylogenetic Tree Inference

ML tree reconstruction was done from all data sets: *M\_Ento*, *M\_Nono*, *M\_Elli*, and *M\_DiCo*, SOS, and SOS<sub>0</sub> (discussed earlier). We estimated evolutionary models for each data set with ModelGenerator v.0.85 (Keane et al. 2006). The



**Fig. 5.** 2D simplex graph. Voronoi cells are areas, in which quartets show predominant or maximal support for either of the three topologies  $T_1$ ,  $T_2$ ,  $T_3$ , or in which quartets show ambiguous support  $T_{12}$ ,  $T_{13}$ ,  $T_{23}$ , and  $T^*$ . For further explanations, refer to Strimmer and von Haeseler (1997, fig. 3). Voronoi cell corresponding to  $T_1$  (blue): quartets show support for (Protura + Diplura) – (Collembola + remaining taxa); Voronoi cell corresponding to  $T_2$  (red): quartets show support for (Protura + Collembola) – (Diplura + remaining taxa); Voronoi cell corresponding to  $T_3$  (yellow): quartets show support for (Diplura + Collembola) – (Protura + remaining taxa); Voronoi cells corresponding to  $T_{12}$ ,  $T_{13}$ ,  $T_{23}$  (gray) do not show clear support for  $T_1$ ,  $T_2$ , and  $T_3$ ; in  $T^*$  all topologies are equally likely.



best fitting model was selected based upon the Akaike Information Criterion (AIC; Akaike 1974). ML trees were inferred with RAxML (Stamatakis 2006), v.7.2.8-ALPHA, HYBRID (Ott et al. 2007; Pfeiffer and Stamatakis 2010) using the CAT model of rate heterogeneity (Stamatakis 2006) and the LG protein substitution matrix (Le and Gascuel 2008). Final tree searches were conducted under the GAMMA model of rate heterogeneity (Yang 1996). Bootstrap analyses were performed with the rapid algorithm (Stamatakis 2006), which also included subsequent searches for the best scoring ML tree. We obtained BS for each node from 1,000 rapid bootstrap replicates, and checked a posteriori if sufficient bootstrap trees were computed using the bootstopping criteria (Pattengale et al. 2010, default settings). ML analyses were conducted on a Linux cluster at the Cologne High Efficient Operating Platform for Science (CHEOPS), Regionales Rechenzentrum Köln (RRZK), using eight nodes with 12 cores each.

After tree inference, we scrutinized our trees for rogue taxa (Aberer et al. 2013; Aberer and Stamatakis 2011, see [supplementary material \[section 3\]](#), [figs. S2 and S4](#), [table S7](#), [Supplementary Material](#) online, for details and results). We removed sequences corresponding to taxa that were identified as rogues from the concatenated alignments and repeated the tree inferences. All trees were edited with Treegraph v.2.0 (Stöver and Müller 2010), and rooted with *Capitella* sp. Data sets are deposited at Dryad: <http://doi.org/10.5061/dryad.mk8p7> (last accessed November 4, 2013).

## Supplementary Material

Supplementary material (sections 1–6), tables S1–S7, and figures S1–S14 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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