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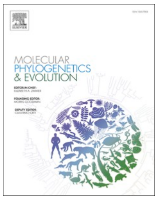

How many long branch orders occur in Chelicerata? Opposing effects of Palpigradi and Opilioacariformes on phylogenetic stability

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ABSTRACT

Excepting a handful of nodes, phylogenetic relationships between chelicerate orders remain poorly resolved, due to both the incidence of long branch attraction artifacts and the limited sampling of key lineages. It has recently been shown that increasing representation of basal nodes plays an outsized role in resolving the higher-level placement of long-branch chelicerate orders. Two lineages have been consistently undersampled in chelicerate phylogeny. First, sampling of the miniaturized order Palpigradi has been restricted to a fragmentary transcriptome of a single species. Second, sampling of Opilioacariformes, a rarely encountered and key group of Parasitiformes, has been restricted to a single exemplar. These two lineages exhibit dissimilar properties with respect to branch length; Opilioacariformes shows relatively low evolutionary rate compared to other Parasitiformes, whereas Palpigradi possibly acts as another long-branch order (an effect that may be conflated with the degree of missing data). To assess these properties and their effects on tree stability, we constructed a phylogenomic dataset of Chelicerata wherein both lineages were sampled with three terminals, increasing the representation of these taxa per locus. We examined the effect of subsampling phylogenomic matrices using (1) taxon occupancy, (2) evolutionary rate, and (3) a principal components-based approach. We further explored the impact of taxon deletion experiments that mitigate the effect of long branches. Here, we show that Palpigradi constitutes a fourth long-branch chelicerate order (together with Acariformes, Parasitiformes, and Pseudoscorpiones), which further destabilizes the chelicerate backbone topology. By contrast, the slow-evolving Opilioacariformes were consistently recovered within Parasitiformes, with certain subsampling practices recovering their placement as the sister group to the remaining Parasitiformes. Whereas the inclusion of Opilioacariformes always resulted in the non-monophyly of Acari with support, deletion of Opilioacariformes from datasets consistently incurred the monophyly of Acari except in matrices constructed on the basis of evolutionary rate. Our results strongly suggest that Acari is an artifact of long- branch attraction.

1. Introduction

Long branch attraction (LBA) refers to a well-characterized phylogenetic artifact in which rapidly evolving lineages (branches) that are distantly related are incorrectly resolved as sister taxa (Bergsten, 2005; Felsenstein, 1978). The symptoms of this artifact include the consistent recovery of counterintuitive groupings that are united only by accelerated evolutionary rate; the placement of one or more rapidly evolving ingroup lineages at the base of the tree near outgroups, which tend to be comparatively poorly sampled; or a combination of both of these. LBA is a form of statistical inconsistency, which prevents overcoming this artifact through quantity of data alone. Several approaches have been proposed to mitigate LBA, such as the use of model-based approaches to phylogeny, the implementation of more sophisticated evolutionary models (e.g., site heterogeneous models) in molecular phylogenetics, intensive taxonomic sampling to “break” long branches, data recoding strategies, omission of rapidly evolving genes or sites, omission of fast-evolving lineages (or substitution with slow-evolving exemplars), and various criteria for data curation. Strategies for mitigating LBA seem to vary in effectiveness from one taxonomic group to another, as a function of species richness, the disparity of evolutionary rates of the taxa in question, the phylogenetic depth of the splits that are targeted for resolution, and the number of long-branch taxa present in the ingroup.

A prerequisite to mitigating LBA is to diagnose the ingroup lineages in which it occurs. Ideally, the number of long-branch taxa is limited to two, as fewer fast-evolving lineages translates to fewer potential

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attractants in the content of this artifact. One of the Gordian knots of the animal tree of life is the basal phylogeny of Chelicerata, the subdivision of arthropods that includes the sea spiders (Pycnogonida), the horseshoe crabs (Xiphosura), and an assemblage of 12 terrestrial orders (Arachnida). The last of these includes at least three long-branch lineages: Pseudoscorpiones (pseudoscorpions or "book scorpions"), Parasitiformes, and Acariformes. Compounding an ancient rapid radiation at the base of Euchelicerata (=Arachnida + Xiphosura), these long-branch taxa have consistently clustered together as basally branching groups in molecular phylogenies of Chelicerata. It is traditionally thought that Parasitiformes and Acariformes together form the clade Acari, and indeed, they are sometimes recovered as sister groups in molecular phylogenies (Howard et al., 2020; Lozano-Fernandez et al., 2019). However, the long branch lengths subtending these taxa, together with inconsistent support for this result across phylogenomic studies (Ballesteros et al., 2019; Ballesteros and Sharma, 2019; Ballesteros et al., 2021a,b), disfavors the monophyly of Acari as a definitive grouping. As a result, the higher-level relationships within Euchelicerata are poorly understood, with recent datasets disputing even the monophyly of Arachnida (Ballesteros et al., 2019; Ballesteros and Sharma, 2019; Ballesteros et al., 2021a,b; Howard et al., 2020; Nolan et al., 2020; Sharma et al., 2014).

In a recent work examining the placement of pseudoscorpions (Ontano et al., 2021), it was shown that pseudoscorpions are reliably placed as the sister group of scorpions in phylogenomic analyses based on BUSCO genes, provided that pseudoscorpions are well-sampled (i.e., that the branch length subtending this group is broken by the sampling of basally branching superfamilies); omitting the representation of basal nodes of this long-branch order resulted in their placement with one or the other long-branch acarine orders instead. The resolution of Pseudoscorpiones + Scorpiones was validated by the discovery that pseudoscorpions share a whole genome duplication with the remaining arachnopulmonates (scorpions, spiders, and three other orders that bear book lungs; Ontano et al. 2021), a result that cannot be reconciled with the alternative placement of pseudoscorpions with the other long-branch orders (as Parasitiformes and Acariformes exhibit no evidence of whole genome duplication; Leite et al., 2016, 2018; Gainett et al., 2021; Ontano et al. 2021). Thus, Ontano et al. (2021) were able to show that taxonomic sampling outperformed other strategies like filtering for dataset occupancy, filtering for evolutionary rate, use of site heterogeneous models, and algorithmic approaches to tree reconstruction in the context of mitigating LBA with respect to the placement of pseudoscorpions.

Regrettably, one lineage missing from that previous work was Palpigradi, the most enigmatic of the arachnid orders. This miniaturized group of arachnids exhibits a mysterious combination of morphological characters that has confounded efforts to place it reliably in arachnid phylogeny on the basis of morphological data, such as the absence of eyes, presence of a sternum, a coxal gland similar in anatomy to that of sun spiders (Solifugae), and a multi-articled flagellum on the posterior terminus (Shultz, 1990, 2007; Weygoldt & Paulus, 1979; ref. Ballesteros et al., 2019). Only one transcriptomic library of a palpigrade (the species *Eukoenenia spelaea*) has ever been produced for analysis in chelicerate phylogeny (Ballesteros et al., 2019), but due to the quality of that dataset (BUSCO completeness: 37%), Palpigradi was not well represented across phylogenomic matrices in that study, and its placement was inconclusive (partial support was obtained for the sister group relationship of palpigrades to Solifugae). Due to the level of missing data for this lineage, it could not be included in the main analyses of Ontano et al. (2021), as these required a specific minimum occupancy threshold per order. At present, it is therefore not known whether the instability of Palpigradi was attributable to missing data in the study of Ballesteros et al. (2019a) or if Palpigradi constitutes a true long-branch taxon. A recent unpublished analysis included a new transcriptome of a different *Eukoenenia* species (Ballesteros et al., 2021a), and thus Palpigradi sampling remains limited to the genus *Eukoenenia*.

Drawing upon the lessons of Ontano et al. (2021), a separate concern for resolving the chelicerate tree of life is the sampling of basal nodes within fast-evolving orders, or ideally, the inclusion of slowly evolving lineages within such orders. One key candidate in this regard is Opilioacariformes, a slowly evolving group of Parasitiformes (Klompen et al. 2007; Pepato et al. 2010). The inclusion of the first opilioacariform library in the study of Ballesteros et al. (2019a) showed that this group exhibited the smallest patristic distance within the Parasitiformes, consistent with previous Sanger dataset-based studies (Klompen et al. 2007; Pepato et al. 2010). Intriguingly, Ontano et al. (2021) were able to show that previously published datasets supporting Acari monophyly (Howard et al. 2020) would instead support Acari diphyly upon the inclusion of that single opilioacariform exemplar, even when analyzed under the same analytical conditions.

To understand how the inclusion of Palpigradi and Opilioacariformes impacts chelicerate relationships (viz. LBA), we endeavored to increase the sampling and data quality of these groups in phylogenomic studies. While most chelicerate orders are represented in recent phylotranscriptomic datasets with at least multiple terminals and genera (Ballesteros et al., 2021a), within Palpigradi, transcriptomes are only available for two species of Eukoeneniidae (both *Eukoenenia*). Here, we focused our efforts on generating a high-quality library of the other family, Prokoeneniidae, for the purpose of breaking the branch subtending the crown group of palpigrades. In addition, we generated a new high-quality library for the genus *Opilioacarus*, bringing the total number of exemplars of each group to three datasets. Here, we show that Palpigradi constitutes a fourth long-branch order of Chelicerata, whose inclusion further destabilizes chelicerate relationships. By contrast, Opilioacariformes has the opposite effect, with its inclusion breaking up the monophyly of Acari, which we show to be an LBA artifact.

2. Materials and methods

2.1. Field collection and sequencing

Specimens of the palpigrade *Prokoenenia wheeleri* and the opilioacariform *Opilioacarus texanus* were hand collected from Cypress Creek Park, Travis County, Austin, Texas, United States (30.438459, −97.874670) on 8–9 January 2020 by A.Z.O., P.P.S., Emily V.W. Setton, and Jesús A. Ballesteros. 20 individuals of *P. wheeleri* and one individual of *O. texanus* were transferred directly to Trizol TriReagent using paintbrushes and stored on ice. RNA extraction, mRNA purification, and library preparation followed our previously published protocols (Ballesteros et al., 2019). Sequencing was performed on an Illumina NovaSeq 6000 platform with a 2 × 150 bp paired end sequencing strategy. Completeness of single-copy BUSCOs (Benchmarking Universal Single Copy Orthologs) were 90.3% for *P. wheeleri* and 96.1% for *O. texanus*, as inferred using the BUSCO-Arach dataset for arachnids derived from OrthoDB v.10 (Kriventseva et al., 2019; Simão et al., 2015; Waterhouse et al., 2018).

The small body size of *P. wheeleri* incurs high risks of environmental contamination in pooled samples. As an additional validation of on-target sequencing, we performed BLASTn searches in the *P. wheeleri* transcriptome for a set of genes previously Sanger-sequenced for this species in the 62-locus dataset of Regier et al. (2010). For all genes recovered in this search, the best BLAST hit was invariably to the *P. wheeleri* data previously generated in the Regier et al. (2010) study.

2.2. Species sampling and orthology inference

We compiled a dataset of 126 chelicerates (3 Palpigradi, 38 Pseudoscorpiones, 12 Scorpiones, 18 Araneae, 6 Pedipalpi, 13 Opiliones, 7 Ricinulei, 3 Xiphosura, 2 Solifugae, 12 Parasitiformes, 10 Acariformes, 2 Pycnogonida) and 15 outgroups (3 Onychophora, 5 Myriapoda, 7 Pancrustacea). This dataset (Table S1) overlaps closely with our recent work (Ontano et al. 2021), with the following modifications: we added libraries for three palpigrades, two Ricinulei, and three parasitiform mites, including two opilioacariform mites (*Neacarus* sp. and *Opilioacarus* sp.). Taxon selection prioritized libraries of high quality and the representation of basal splits in all major groups (Ballesteros et al., 2019, 2021; Benavides et al., 2019; Fernández et al., 2017, 2018; Santibáñez-López et al., 2019, 2020; Sharma et al., 2015).

Candidate ORFs were identified in transcripts using TransDecoder (Haas et al., 2013). Loci selected for phylogenomic analysis consisted of the subset of 2934 Benchmarked Universal Single Copy Orthologs identified for Arachnida (BUSCO-Arach) derived from OrthoDB v.10 (Kriventseva et al., 2019; Simão et al., 2015; Waterhouse et al., 2018). Each library was analyzed with the OrthoDB pipeline to identify available homologs of 2934 arachnid-specific BUSCO genes. Duplicated BUSCOs were discarded to retain only validated, single-copy loci. We further filtered loci using a taxon decisiveness criterion, such that every retained gene had to sample at least one member of each higher-level lineage (chelicerate orders; Mandibulata; Pancrustacea; Onychophora) to be included in the dataset. This filtering resulted in an initial set of 1024 BUSCO genes. Multiple sequence alignment was performed using MAFFT 7.3.8 (*-anysymbol –auto*; Katoh & Standley, 2013). Gap-rich regions were masked with trimAl 1.2 (*-gappyout*; Capella-Gutiérrez et al., 2009) and alignment coverage verified and sanitized with Al2Phylo (*-m 50 -p 0.25 -t 20*; Ballesteros & Hormiga, 2016).

2.3. Phylogenomic subsampling

We investigated three approaches to ordered phylogenomic subsampling. First, we generated matrices of 200, 400, and 600 loci based on taxon occupancy. Second, we generated three matrices of 200, 400, and 600 loci based on mean percent pairwise sequence identity (MPSI), a proxy for evolutionary rate. Third, we applied a recently developed principal components-based method that accounts for multiple metrics of phylogenetic usefulness (*sortR*; Mongiardino Koch, 2021).

sortR requires a resolved species tree *a priori* for the computation of Robinson-Foulds distances for each gene tree. However, given the marked conflict at the base of the chelicerate tree of life, within some chelicerate orders, as well as within some outgroups (e.g., Myriapoda), we endeavored to limit the influence of the species tree on the ranking of phylogenetically useful genes. We therefore supplied a species tree wherein all nodes were collapsed except for higher-level splits that are robustly supported across analyses (e.g., monophyly of chelicerate orders, Tetrapulmonata, Pedipalpi, Euchelicerata, Chelicerata, Mandibulata, Pancrustacea, and Arthropoda). This pipeline recovered an axis of phylogenetic usefulness (PC2); as before, matrices of 200, 400, and 600 loci were generated from the ranking.

2.4. Phylogenomic inference

Tree topologies for individual loci and for concatenated datasets were computed with IQ-TREE v.1.6.11 (Chernomor et al., 2016; Nguyen et al., 2015), coupled with model selection of substitution and rate heterogeneity based on the Bayesian Information Criterion (Kalyaanamoorthy et al., 2017) and 1000 ultrafast bootstraps to assess branch support (*-m MFP -mset LG, JTT, WAG -st AA -bb 1000*; Hoang et al., 2018). We additionally performed model selection under the posterior mean site frequency (PMSF), a mixture model that approximates the CAT model in a maximum likelihood framework (Lartillot & Philippe, 2004; Wang et al., 2018). Analyses were performed using the LG + C20 + F + Γ model.

2.5. Taxon deletion experiments

For each of the nine matrices previously generated, we performed the following deletions of taxa and recomputed the tree topology under models for the corresponding full dataset. First, to assess the impact of Opilioacariformes (the slowly evolving putative sister group of the remaining Parasitiformes; Pepato et al., 2010) in the phylogeny, we removed Opilioacariformes from each dataset. Second, to evaluate the topological stability of four putative long-branch taxa (Acariformes, Parasitiformes, Palpigradi, and Pseudoscorpiones), we deleted all long-branch taxa but Palpigradi and computed the resulting trees. Lastly, we treated Opilioacariformes as separate from Parasitiformes, and deleted all long-branch taxa except Palpigradi and Opilioacariformes, and computed the resulting trees.

3. Results

3.1. Phylogenetic analyses under varying matrix construction criteria

Data matrices constructed using taxon occupancy thresholds (200-, 400-, and 600-most complete loci) resulted in the representation of 83%, 78%, and 73% of the 141 terminals, respectively. Data matrices constructed using evolutionary rate thresholds (200-, 400-, and 600-slowest evolving loci) resulted in mean sequence identity values of 73.7%, 65.0%, and 58.3% (across the entire matrix), respectively.

Maximum likelihood (ML) searches of these nine matrices recovered inconsistent phylogenetic placement of Palpigradi across analyses (Fig. 1). Matrices constructed based on taxon occupancy recovered the relationship Palpigradi + Pseudoscorpiones (BS = 91–96%) or the relationship Palpigradi + Parasitiformes with poor support (BS = 74%) (Fig. 1a). In all matrices built using taxon occupancy, pseudoscorpions were recovered as part of a grade toward the base of Euchelicerata, rather than as part of a monophyletic Arachnopulmonata. Of these three matrices, only the most complete matrix (200 loci) recovered Opilioacariformes as the sister group to the remaining Parasitiformes. The inclusion of more loci recovered a nested position with Opilioacariformes + Ixodida as sister group to Mesostigmata.

Matrices constructed based on evolutionary rate (Fig. 1b) recovered Palpigradi in a grade with Acariformes at the base of Euchelicerata (BS = 88–99%). The 200- and 400-slowest evolving loci matrices were able to recover the monophyly of Arachnopulmonata and Panscorpiones, albeit with waning support for Panscorpiones (BS = 72%) in the 400-slowest evolving loci matrix. In the 600-slowest evolving loci matrix, pseudoscorpions were recovered as the sister group of the remaining arachnopulmonates, though this relationship was not supported (BS = 56%). In this family of matrices, only the 200-slowest evolving loci matrix was able to recover the placement of Opilioacariformes as the sister group of the remaining Parasitiformes (BS = 93%), with the inclusion of noisier genes resulting in the nested placement of Opilioacariformes as the sister group of Ixodida.

Matrices constructed based on PC2 of the *sortR* pipeline recovered the relationships Palpigradi + Ricinulei (BS = 96%), Palpigradi + Solifugae (BS = 79%), or Palpigradi + Parasitiformes (BS = 79%), as a function of increasing matrix size. ML analysis of the 200 most “useful” loci matrix resulted in the recovery of Panscorpiones (BS = 95%) and Arachnopulmonata (BS = 100%), as well as the placement of Opilioacariformes as the sister group of the remaining Parasitiformes (BS = 100%). The addition of less useful (noisier) genes resulted in the nested placement of Opilioacariformes, as well as loss of support for Panscorpiones, with eventual dissolution of Arachnopulmonata in the 600 most useful loci matrix. The monophyly of Acari was not recovered in any partitioned model analysis.

Given the rare genomic change uniting Arachnopulmonata (Ontano et al. 2021), matrix construction criteria were compared directly on the basis of pseudoscorpion placement (Fig. 2).

3.2. Taxon deletion experiments

Taxon deletion experiments followed one of three schemes: removal of Opilioacariformes (Fig. 3), removal of all long-branch chelicerate orders except Palpigradi (Fig. 4), or removal of all long-branch orders except both Palpigradi and Opilioacariformes (Fig. 5).

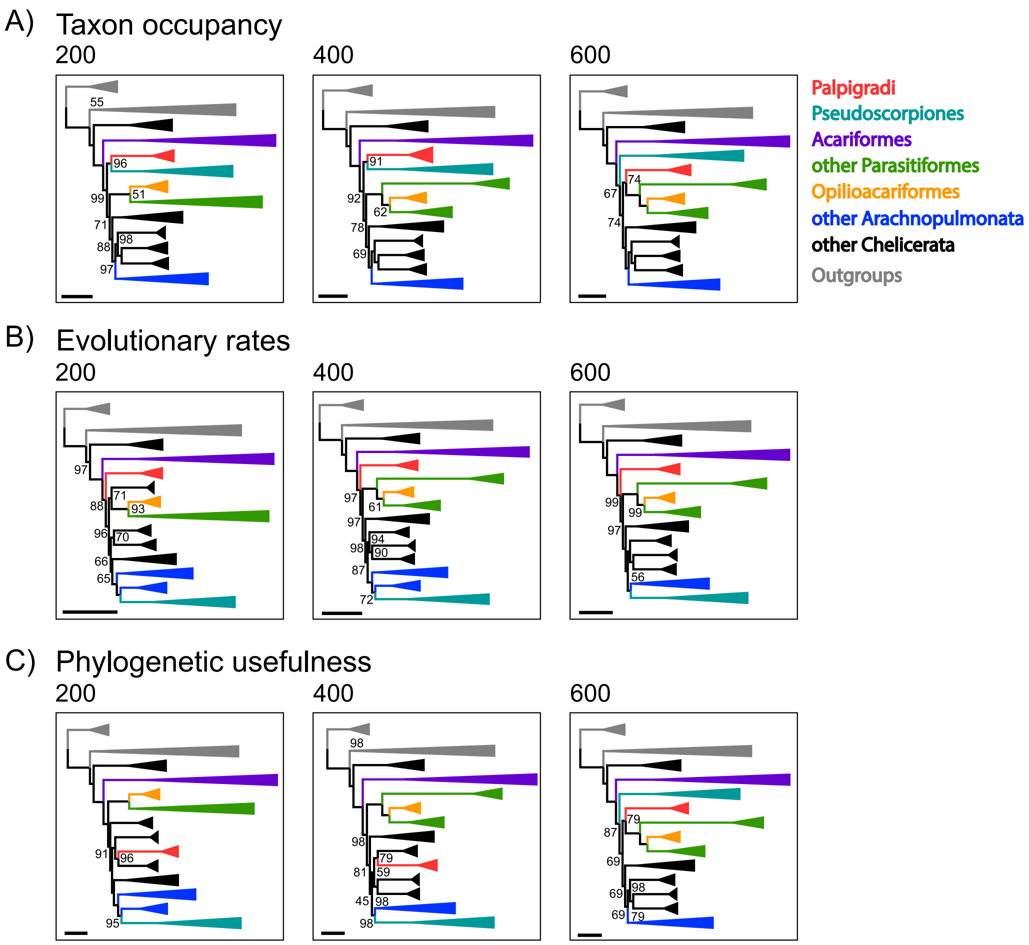

Fig. 1. Maximum likelihood trees constructed from matrices of 200, 400, and 600 loci, selected under alternative optimality criteria. (a) Ordered by taxon occupancy. (b) Ordered by evolutionary rate. (c) Ordered by phylogenetic usefulness (output of *sortR*). Colors correspond to legend on the right. Ultrafast bootstrap support values under 100% are labeled next to their corresponding node; unlabeled nodes are maximally supported. Scale bar: 0.2. Note the placement of Opilioacariformes within Parasitiformes as a function of matrix composition.

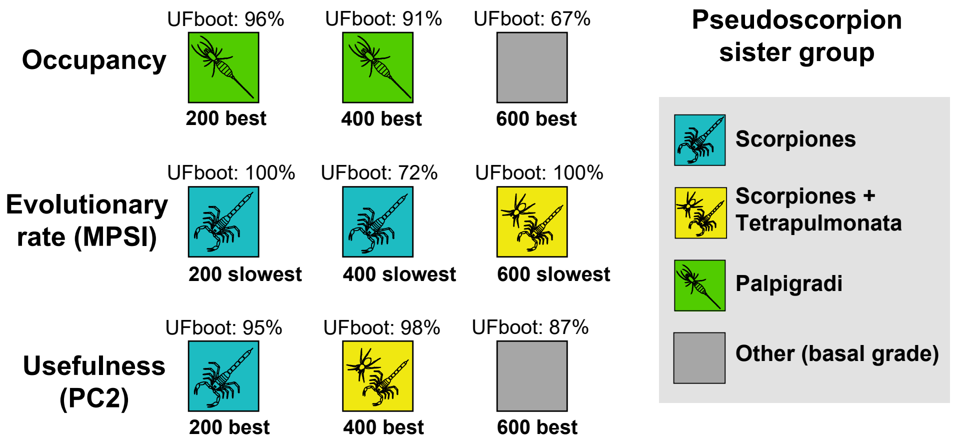

Fig. 2. Sensitivity plot of pseudoscorpion placement in maximum likelihood analyses shown in Fig. 1, with corresponding ultrafast bootstrap support values.

With the removal of Opilioacariformes (Fig. 3), matrices constructed under taxon occupancy and phylogenetic usefulness criteria (PC2) recovered Acari as monophyletic (BS = 88–99% for occupancy; BS = 96–100% for PC2), whereas matrices constructed through MPSI consistently recovered the poorly supported relationship Palpigradi + Parasitiformes (BS = 51–70%). The removal of Opilioacariformes further destabilized the placement of Pseudoscorpiones and decreased support for arachnopulmonate monophyly (compare support values to Fig. 1c). Matrices constructed under the criterion of evolutionary rate (Fig. 3b) consistently recovered Acari as diphyletic, with Parasitiformes recovered as the sister group of Palpigradi with low support (BS = 51–70%).

The removal of all long-branch orders except Palpigradi (Fig. 4) did not have a strong effect on the placement of Palpigradi. In the absence of other long-branch orders, Palpigradi are recovered as sister to all other euchelicerates, except in the matrix constructed using the 200 most useful loci (i.e., highest values for PC2; Fig. 4c), which recovered the relationship Palpigradi + Ricinulei (BS = 89%).

In analyses that included Opilioacariformes and Palpigradi in the absence of the remaining long-branch taxa (Fig. 5), matrices constructed

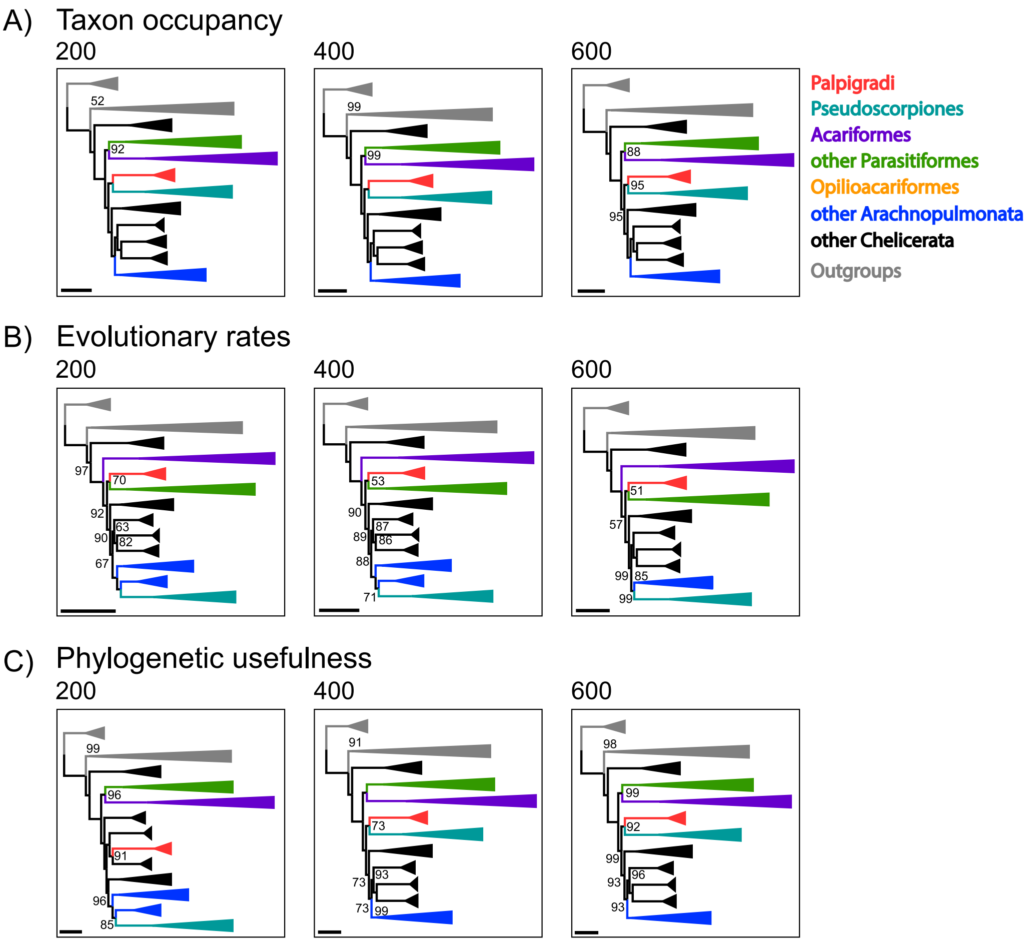

Fig. 3. Taxon deletion experiments removing Opilioacariformes result in the artifactual monophyly of Acari. Maximum likelihood trees are shown with same arrangement of matrix optimality criteria as in Fig. 1. Colors correspond to legend on the right. Ultrafast bootstrap support values under 100% are labeled next to their corresponding node; unlabeled nodes are maximally supported. Scale bar: 0.2.

using taxon occupancy and evolutionary rate recovered the relationship Parasitiformes + Xiphosura (BS = 93–99%), except in the matrix incorporating the 600-slowest evolving loci. This analysis, along with all matrices constructed on the criterion of phylogenetic usefulness (Fig. 5c), recovered the relationship Parasitiformes + Solifugae (BS = 100% for evolutionary rate-based matrices; BS = 59–100% for phylogenetic usefulness-based matrices).

3.3. *Analyses with site heterogeneous model*

We performed the same family of analyses above, but with site heterogeneous model implementations for maximum likelihood. Tree topologies were therefore computed under the PMSF model (LG + C20 + F + Γ). Results differed minimally from partitioned analyses and are not discussed in detail here; arachnid monophyly was never recovered in any analysis. In analyses with all taxa, Palpigradi were recovered more frequently in a basal grade in comparison to partitioned model approaches (Fig. S1). Arachnopulmonata was maximally supported across all matrices constructed based on evolutionary rate (Fig. S1b) and phylogenetic usefulness (Fig. S1c), but pseudoscorpions were recovered as sister to all other arachnopulmonates in all these analyses except for the 200-slowest evolving loci matrix, which recovered Panscorpiones (BS = 86%; Fig. S1b).

Taxon deletion experiments under the PMSF model also reflected similar results (Figs. S2-S4). In analyses with all long-branch taxa except Palpigradi removed (Fig. S3), we recovered a placement of Palpigradi in a basal grade, except for the matrix constructed from the 200 most useful (i.e., highest PC2) loci matrix, where they were recovered as the sister group to the clade Ricinulei + Xiphosura (BS = 98%). In analyses with Opilioacariformes removed, the monophyly of Acari was never recovered (Fig. S2), paralleling the outcomes of partitioned model analyses.

4. Discussion

4.1. *Palpigradi: The fourth long-branch order of Chelicerata*

Previous analyses of chelicerate phylogeny had applied distributions of patristic distances and taxon deletion experiments to explore the stability of the arachnid orders (Sharma et al., 2014; Ballesteros and Sharma, 2019). These works had not been able to sample either Palpigradi or Opilioacariformes, and thus concluded that at least three orders exhibited clearly problematic trends of accelerated evolution: Acariformes, Parasitiformes, and Pseudoscorpiones. Due to the quality of the only available palpigrade library, Ballesteros et al. (2019a) were not able to infer whether the instability of *Eukoenenia spelaea* in a phylotranscriptomic dataset was attributable to poor taxonomic sampling, missing data, or other systematic artifacts.

Here, we generated the highest quality dataset of Palpigradi to date, adding this to the existing pair of transcriptomes for two *Eukoenenia*. The sampling of both Prokoeneniidae and Eukoeneniidae ensures the representation of the basal-most node in crown-group Palpigradi, given that only these two extant families are known and previous work has shown them to be reciprocally monophyletic (Giribet et al., 2014). In addition, the high quality of the *P. wheeleri* library, together with our decisiveness

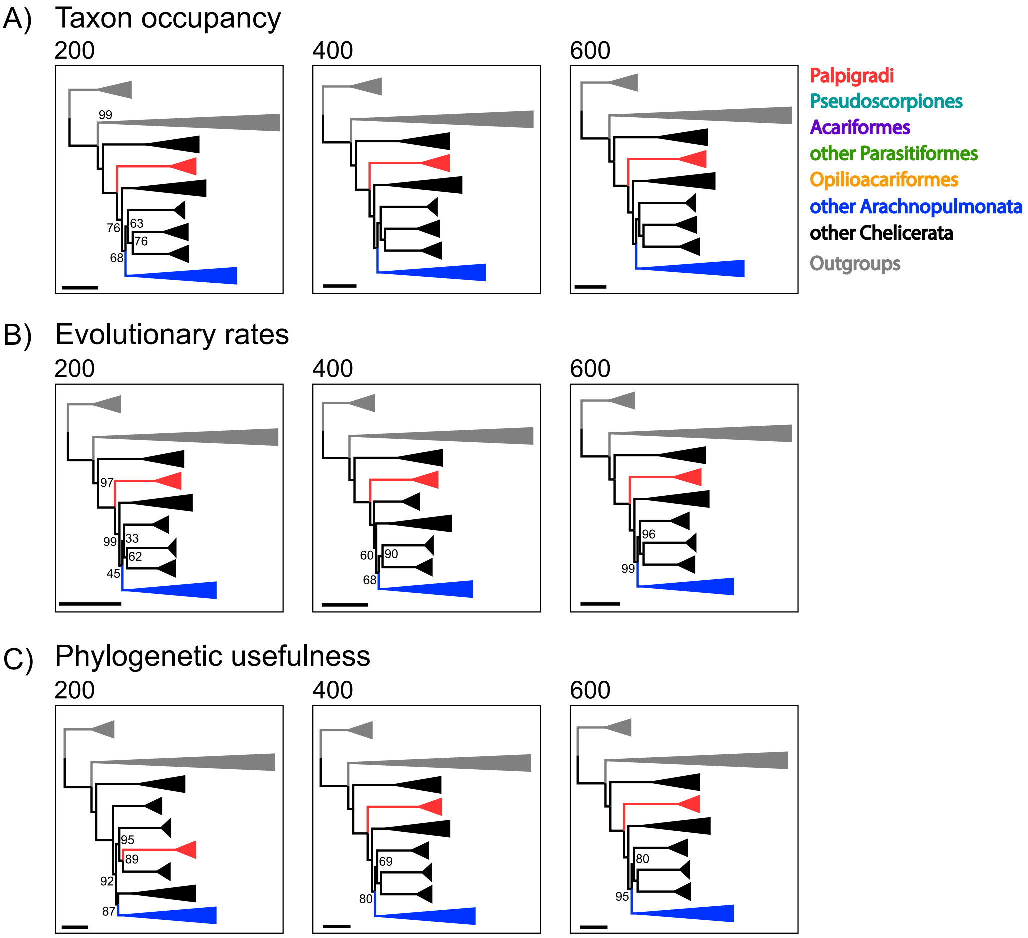

Fig. 4. Taxon deletion experiments that remove all long-branch orders except Palpigradi do not mitigate phylogenetic instability of this lineage. Maximum likelihood trees are shown with same arrangement of matrix optimality criteria as in Fig. 1. Colors correspond to legend on the right. Ultrafast bootstrap support values under 100% are labeled next to their corresponding node; unlabeled nodes are maximally supported. Scale bar: 0.2.

criterion (i.e., retaining only genes that sampled at least one exemplar of each chelicerate order, as well as major outgroup lineages), limited the impact of missing data or uninformative orthogroups for inferring higher-level relationships.

Our analyses of chelicerate relationships with these augmented matrices revealed clear evidence of instability in palpigrade placement, as a function of matrix assembly criterion (Figs. 1, S1). Palpigradi typically clustered with pseudoscorpions (Fig. 1a, S1a), formed a grade with Acariformes at the base of Euchelicerata (Fig. 1b, S1b), or was recovered as sister group to other unstable groups like Parasitiformes or Ricinulei (Fig. 1c, S1c). The instability exhibited by this taxon, together with its clustering near the base of the tree with other long-branch orders, are strongly suggestive of an LBA artifact.

To corroborate the instability of Palpigradi across datasets, we undertook a separate analyses wherein we added the three palpigrade libraries to the G_3 matrix of Ontano et al. (2021), which consisted of 693 loci (70% taxon occupancy threshold) assembled with an older generation of arthropod-specific BUSCO genes. This matrix, which sampled all pseudoscorpion superfamilies, was previously shown to be able to recover Panscorpiones (BS = 81%) and Arachnopulmonata (BS = 81%) under either partitioned or site heterogeneous model approaches. Upon addition of Palpigradi to this dataset, Pseudoscorpiones were drawn to the base of the tree as the sister group of Acariformes (BS = 85%), with the four long-branch orders (Acariformes, Pseudoscorpiones, Palpigradi, Parasitiformes) forming a grade at the base of Euchelicerata (Fig. S5).

These results suggest that the instability of Palpigradi is not attributable to subsampling matrices smaller than ca. 700 genes, nor to properties of the BUSCO-Arach loci. We infer that Palpigradi constitutes a fourth long-branch arachnid order, whose inclusion in chelicerate phylogenomic datasets only further destabilizes the basal euchelicerate topology. This inference is consistent with the observation that the four long-branch chelicerate taxa all exhibit different degrees of miniaturization, and, in the case of many groups of Acariformes and Parasitiformes, a parasitic lifestyle. Both of these evolutionary phenomena are associated with rapid evolutionary rates, aberrant patterns of genome evolution, and long-branch artifacts throughout Metazoa, as epitomized by LBA artifacts impacting the relationships of Tardigrada and Nematoda (Borner et al., 2014; Laumer et al., 2019).

While taxonomic sampling has been shown to outperform other strategies to resolving LBA artifacts in pseudoscorpions, this strategy may have limited effectiveness for Palpigradi, because the basal-most node in crown-group palpigrades has already been sampled in the present matrix. Adding more exemplars of either palpigrade family may have little effect in breaking the branch subtending this node, if these families are indeed systematically valid and reciprocally monophyletic. New insights from rare genomic changes and the incidence of shared genome duplications may inform the placement of this enigmatic lineage, with particular emphasis on testing an older notion that palpigrades are closely related to Tetrapulmonata (but see Seiter et al., 2021).

4.2. Inclusion of the slowly evolving Opilioacariformes refutes Acari monophyly

The monophyly of Acari is another controversial topic in arthropod

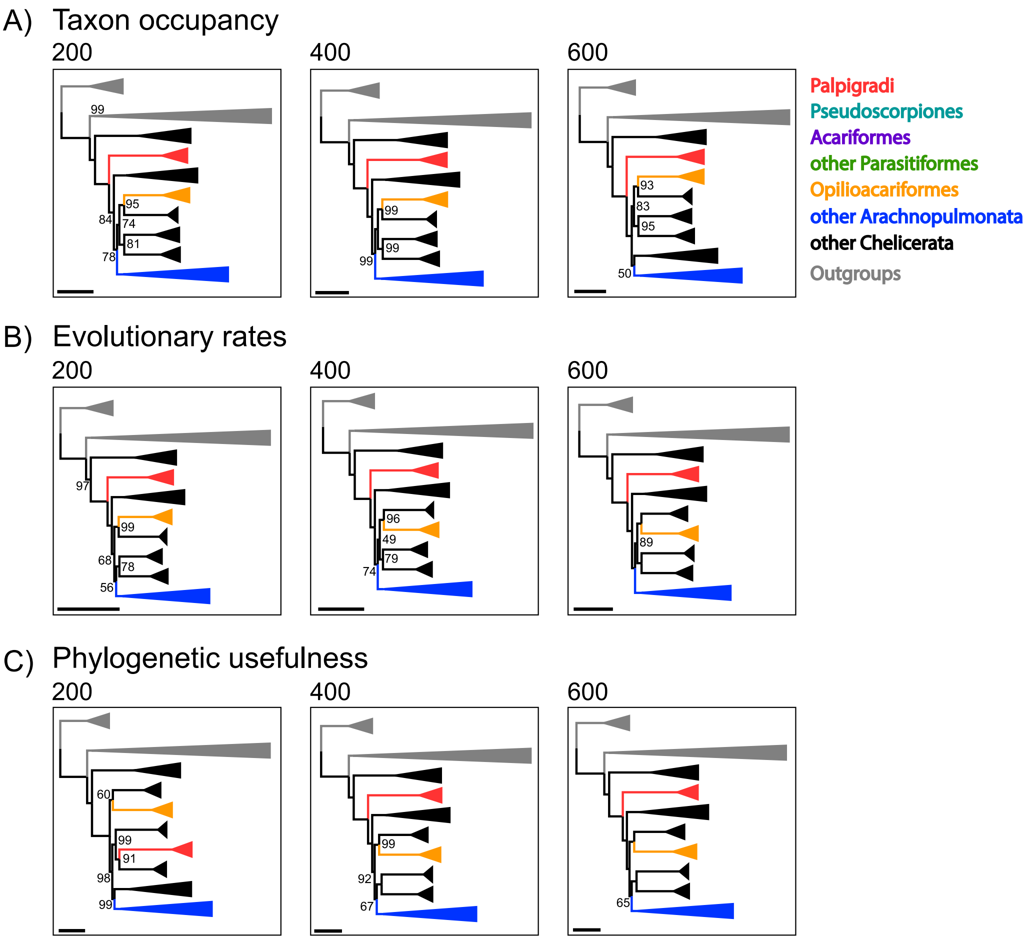

Fig. 5. Taxon deletion experiments retaining only Opilioacariformes and Palpigradi, but no other long-branch taxa. Maximum likelihood trees are shown with same arrangement of matrix optimality criteria as in Fig. 1. Colors correspond to legend on the right. Ultrafast bootstrap support values under 100% are labeled next to their corresponding node; unlabeled nodes are maximally supported. Scale bar: 0.2.

phylogenetics. Various analyses of morphological data have supported the sister group relationship of Acariformes + Parasitiformes (Shultz, 1990, 2007; but see Dunlop et al., 2012; Pepato et al., 2010). Molecular phylogenies have recovered variable support for this relationship (Ballesteros et al., 2019; Ballesteros and Sharma, 2019; Giribet et al., 2001; Howard et al., 2020; Lozano-Fernandez et al., 2019; Masta et al., 2009; Regier et al., 2010; Sharma et al., 2014; Wheeler & Hayashi, 1998), which suspiciously resembles an LBA artifact, owing to the long patristic distances exhibited by commonly studied exemplars of both groups. One exception to this trend is Opilioacariformes, a rarely encountered and slowly evolving group of Parasitiformes. The sole opilioacariform library sequenced to date exhibited a comparatively short patristic distance across datasets, suggesting that this free-living (non-parasitic) taxon did not share the rapid evolutionary rates and genomic rearrangements observed in many parasitiform genomes (e.g., Hoy et al., 2016).

Upon expanding the sampling of opilioacariform datasets to three genera, we never recovered the monophyly of Acari across our analyses (Fig. 1, S1). In taxon deletion experiments under partitioned model analyses, the removal of Opilioacariformes alone was sufficient to recover Acari monophyly in most datasets (Fig. 3a, 3c). These results closely parallel a previous analysis by Ontano et al. (2021), who added one opilioacariform and one palpigrade library to the analyses of Howard et al. (2020), in order to test the claims that these datasets could recover monophyly of Arachnida and Acari. Ontano et al. (2021) were able to show that the addition of just two phylogenetically significant taxa to those datasets was sufficient to collapse support for both arachnid and acarine monophyly (Figs. S2 and S3 of Ontano et al., 2021). Moreover, in taxon deletion experiments under site heterogeneous models, even the removal of Opilioacariformes did not render Acari monophyletic (Fig. S2).

These results strongly suggest that the monophyly of Acari reflects another LBA artifact in chelicerate phylogeny. The correspondences in the mouthparts and body plans of Acariformes and Parasitiformes therefore likely reflect morphological convergence rather than homologies. Future efforts to resolve the placement of these diverse groups must focus on expanding the sampling of phylogenetically significant groups that break long branches and potentially exhibit lower evolutionary rates. Examples of key targets for future interrogation of acarine relationships in phylogenomic studies include Holothyrida (Parasitiformes) and several groups of the “endeostigmatan” mites (Acariformes), such as the basally branching families Nanorchestidae and Alycidae (Klimov et al., 2018).

4.3. Phylogenetic usefulness versus evolutionary rate in chelicerate phylogeny

Beyond assessing the effects of sampling palpigrades and opilioacariforms in chelicerate phylogeny, we assessed competing strategies for locus selection as antidotes to LBA artifacts. Reducing missing data, with emphasis on clade-specific patterns of missing genes (i.e., rows in phylogenomic matrices), has been argued to be important for phylogenetic accuracy (Roure et al., 2013). In the specific case of LBA, matrix construction using slowly evolving genes (either through filtering out noisy loci, saturated sites, or recoding strategies) has been argued to be an effective solution for reducing artifactual grouping of fast-evolving taxa. A more comprehensive strategy to subsampling genes for high phylogenetic signal and low noise was recently proposed by Mongiardino Koch (2021); *sortR* makes use of a principal components-based approach that aims to maximize metrics of phylogenetic signal (e.g., Robinson-Foulds distance from a species tree; bootstrap values on gene trees; see also Salichos and Rokas, 2013), while minimizing metrics of noise (e.g., root-to-tip variance; saturation; compositional heterogeneity). This promising approach offers a more reliable and reproducible means of subsampling loci to construct matrices of reasonable size for computationally demanding approaches like phylogenomic dating. For well-behaved datasets, the composite metric of phylogenetic usefulness (principal component 2) has been shown to outperform subsampling by evolutionary rate (principal component 1), with exceptions in the cases of ancient and complex radiations (e.g., Hexapoda; Phasmatodea; Mongiardino Koch, 2021).

We brought all three strategies to bear on the higher-level relationships of Chelicerata, one of two nodes explicitly mentioned by Mongiardino Koch (2021) as an undesirable test case for reason of controversial relationships (*sortR* ideally requires a resolved species tree for calculation of RF distances, though this requirement can be circumvented by collapsing controversial nodes, as performed herein). Given the extensive discordance of signal across datasets at the base of Euchelicerata, we used the placement of pseudoscorpions as our benchmark for phylogenetic accuracy (Fig. 2), as the membership of this long-branch order within Arachnopulmonata is strongly substantiated by rare genomic changes (Ontano et al. 2021).

Of the three strategies, we found subsampling loci for taxon occupancy to be the least effective strategy in overcoming LBA viz. the placement of pseudoscorpions (Fig. 1a). No matrix constructed for optimizing taxon occupancy was able to recover Panscorpiones or Arachnopulmonata, either under partitioned model or site heterogeneous model analyses (Fig. S1a). Subsampling by evolutionary rate was the most effective strategy for recovering pseudoscorpions within Arachnopulmonata, with addition of noisier (i.e., faster-evolving) genes causing pseudoscorpions to be pulled out of Panscorpiones and towards the root of arachnopulmonates (Fig. 1b, S1b). Nevertheless, analyses of all matrices constructed on the basis of evolutionary rate were able to recover Arachnopulmonata (with pseudoscorpions either sister group to scorpions or to the remaining arachnopulmonates).

Subsampling by phylogenetic usefulness was of intermediate effectiveness for recovering Arachnopulmonata. For both partitioned model and site heterogeneous model analyses, only the 200 most useful genes were able to recover Panscorpiones and Arachnopulmonata; the 400 most useful genes recovered Arachnopulmonata, but not Panscorpiones; and the 600 most useful genes recovered pseudoscorpions in a basally branching position near the root of Euchelicerata (Fig. 1c, S1c). These results echo the conclusion of Mongiardino Koch (2021) that subsampling by phylogenetic usefulness may not be a universally effective strategy for ancient rapid radiations that include taxa with high heterogeneity of evolutionary rates.

We additionally observed the phenomenon of LBA artifacts within long-branch taxa. Within Parasitiformes, Opilioacariformes were resolved as the sister group to the remaining Parasitiformes (paralleling the traditional placement of this group, based on Pepato et al., 2010) only in the smallest matrices (the 200 most complete loci; the 200 slowest-evolving loci; and the 200 most useful loci; Figs. 1, S1). The addition of noisier genes, under any of the three criteria for matrix construction, destabilized this topology and recovered the long-branch Mesostigmata as the sister group to the remaining Parasitiformes. We postulate that future efforts to address the relationships within Acariformes and Parasitiformes must take into account the possibility that asymmetric rates of evolution in nested lineages may further exacerbate LBA artifacts in these groups.

5. Conclusion

We showed that Palpigradi and Opilioacariformes have opposing effects on chelicerate phylogeny. Palpigradi is demonstrably an unstable taxon that destabilizes interordinal chelicerate relationships, despite high data occupancy and the sampling of the deepest node within the palpigrade crown-group. Opilioacariformes are a slowly evolving group of Parasitiformes and their inclusion invariably drives the dissolution of Acari, suggesting that Acari monophyly reflects another LBA artifact in chelicerate phylogeny. In addition to expanding taxonomic sampling (Ontano et al., 2021), subsampling with slowly evolving genes may be an effective solution to mitigating LBA artifacts in chelicerate phylogeny (Ballesteros et al., 2021a).

CRediT authorship contribution statement

Andrew Z. Ontano: Methodology, Investigation, Formal analysis. **Hugh G. Steiner:** Formal analysis. **Prashant P. Sharma:** Conceptualization, Methodology, Data curation, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2021.107378.

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