Sources of Signal in 62 Protein-Coding Nuclear Genes for Higher-Level Phylogenetics of Arthropods

Jerome C. Regier1,2,3, Andreas Zwick3,4,5

1 Institute for Bioscience and Biotechnology Research, University of Maryland, College Park, Maryland, United States of America, 2 Department of Entomology, University of Maryland, College Park, Maryland, United States of America, 3 Center for Biosystems Research, University of Maryland Biotechnology Institute, College Park, Maryland, United States of America, 4 Entomology, State Museum of Natural History, Stuttgart, Germany

Abstract

Background: This study aims to investigate the strength of various sources of phylogenetic information that led to recent seemingly robust conclusions about higher-level arthropod phylogeny and to assess the role of excluding or downweighting synonymous change for arriving at those conclusions.

Methodology/Principal Findings: The current study analyzes DNA sequences from 68 gene segments of 62 distinct protein-coding nuclear genes for 80 species. Gene segments analyzed individually support numerous nodes recovered in combined-gene analyses, but few of the higher-level nodes of greatest current interest. However, neither is there support for conflicting alternatives to these higher-level nodes. Gene segments with higher rates of nonsynonymous change tend to be more informative overall, but those with lower rates tend to provide stronger support for deeper nodes. Higher-level nodes with bootstrap values in the 80% - 99% range for the complete data matrix are markedly more sensitive to substantial drops in their bootstrap percentages after character subsampling than those with 100% bootstrap, suggesting that these nodes are likely not to have been strongly supported with many fewer data than in the full matrix. Data set partitioning of total data by (mostly) synonymous and (mostly) nonsynonymous change improves overall node support, but the result remains much inferior to analysis of (unpartitioned) nonsynonymous change alone. Clusters of genes with similar nonsynonymous rate properties (e.g., faster vs. slower) show some distinct patterns of node support but few conflicts. Nonsynonymous change is shown to contribute little, if any, phylogenetic signal to the support of higher-level nodes, but it does contribute nonphylogenetic signal, probably through its underlying heterogeneous nucleotide composition. Analysis of seemingly conservative indels does not prove useful.

Conclusions: Generating a robust molecular higher-level phylogeny of Arthropoda is currently possible with large amounts of data and an exclusive reliance on nonsynonymous change.

Introduction

The robust resolution of higher-level arthropod phylogeny has been a challenging problem, as evidenced by numerous publications with alternative proposals of relationships [1-10]. However, a recent molecular report [11] describes fully resolved relationships within and among four all-inclusive, extant arthropod clades — Pancrustacea, Myriapoda, Euchelicerata, and Pycnogonida — with generally high levels of node support, with some exceptions, particularly inside Euchelicerata (redrawn in Figure 1 of this report; see also Table 1 and Materials & Methods for character set definitions used in this and previous reports). The apparent success of this study is likely due to its relatively broad taxon sample (75 arthropod spp. from all major lineages +5-10 diverse outgroup spp.), large data matrix (up to ca. 40 kilobase pairs / taxon from 62 protein-coding nuclear genes), and focus on appropriate methodologies (e.g., likelihood analyses under a codon model and under models that are informed by nonsynonymous change). Reassuringly, high node support is a general feature across a broad range of analytical methods and character codings. Of perhaps greatest taxonomic interest because of their relative novelty are six newly named groups within Pancrustacea (i.e., Altocrustacea, Vericrustacea, Multicrustacea, Comumnstraca, Miracrustacea, Xenocardia; see Figure 1) plus a group within Myriapoda (i.e., Symphylla + Pauropoda) that receives strong bootstrap support (i.e., ≥80%). While analyses of nucleotides, codons, and amino acids all recover these seven groups in their maximum likelihood topologies, analysis of amino acids is unique in that support for six of the seven is significantly lower than with other approaches, in which bootstrap support is always strong. In that report, it was suggested that the failure of amino acid models to distinguish two clusters of serine codons, standardly called Ser1 (TCN) and Ser2 (AGY), is a cause of lower node support, rather than any problem specific to the nucleotide-based analyses, and this has now been further supported (Zwick, Regier & Zwickl, in preparation). Thus, all analytical approaches now appear to be in close agreement.

Citation: Regier JC, Zwick A (2011) Sources of Signal in 62 Protein-Coding Nuclear Genes for Higher-Level Phylogenetics of Arthropods. PLoS ONE 6(8): e23408.

doi:10.1371/journal.pone.0023408

Copyright: © 2011 Regier, Zwick. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: regier@umd.edu (JCR); andreas.zwick@smns-bw.de (AZ)
However, this amino acid issue, among others, highlights the important further question as to what in particular provides the supportive and conflicting signals that determine node support for the higher-level arthropod clades.

Taxon sampling is clearly important [12,13]. No pancrustacean groups other than Pancrustacea itself are strongly supported when taxon sampling is reduced from 80 to 15 species, even when the taxa are represented by an identical gene sample and are similarly analyzed [5,11].

Features of gene analysis are also important in assessing phylogenetic informativeness. For example, while it is commonly acknowledged that not all genes are equally informative, what is to be made of the finding that six of the seven arthropod nodes mentioned above are never recovered with strong bootstrap support by any of the 62 genes (see Supplementary Table 3 in [11]). A reasonable suggestion would be that their strong node support in the combined-gene analyses results from the cumulative weak signal of multiple genes. This hypothesis raises at least two important questions: 1) How many data are needed to resolve nodes with strong support [14]? and 2) How are we to know whether the cumulative bootstrap signal is actually phylogenetic signal, given the increased sensitivity of large data sets to systematic error, despite their decrease in stochastic error [15–18]?

A second feature of gene analysis is that inclusion of more rapidly evolving genes can result in relatively lower node support values for higher-level groupings [19–24]. For example, it was found that when the 10 most rapidly evolving genes were deleted from a 13-taxa data matrix, bootstrap support for Hexapoda (represented by 2 spp.) increased from <50% to 79% and for Malacostraca + Copepoda (represented by 2 spp., included in what is now called Multicrustacea) from 68% to 97% [5]. But, does this correlation permit a practical generalization for designing data sets for arthropod phylogeny?

A third feature of gene analysis is that synonymous change can have a deleterious effect on inferring higher-level phylogeny [25–28], although the appropriateness of their exclusion is controversial [29–33]. At least in part, this results from increased compositional heterogeneity of its underlying characters relative to those driving nonsynonymous change [34,35]. Even modest amounts of synonymous change (e.g., the amount contributed by some first-codon-position characters in a standard first-+ second-codon analysis) can result in strong node support for incorrect groups (see Figure 1 in [5]). Such compositional effects are widely acknowledged and documented (e.g., [36–51; but see 52]), but even more widely ignored in practice, probably because most readily available software packages do not address the problem. Recently, there has been an increased interest in directly accounting for compositional changes in phylogenetic analyses [53–56], but for now this remains a work in progress. Implementation of a codon model can indirectly diminish the contribution of compositional heterogeneity because synonymous change occurs relatively rapidly [57,11 but see 58,59]. A conceptually similar, but much less computationally demanding, approach is to partition data and apply separate models or parameters [26,28,32,60]. When synonymous and nonsynonymous changes are enriched in separate bins, the synonymous change becomes effectively down-weighted, an approach that has been shown in Lepidoptera to give results similar to implementation of a codon model, with both approaches showing improvement over likelihood analysis under the unperturbed GTR + gamma + Inv model [61,62]. The effect of partitioning on the arthropod data set of Regier et al. (2010) [11] is documented for the first time in the current report.

### Table 1. Definitions of degen1 coding and of character sets.

| Character Set | Description |
|---------------|-------------|
| nt123         | A data set consisting of all nucleotide characters (after exclusion of a mask) in a multisequence alignment. |
| nt1, nt2, nt3 | A data subset consisting of all and only first-, second-, or third-codon-position characters, respectively, in a multisequence alignment. |
| LRA1nt1       | A data subset consisting of all and only those nt1-characters that encode one or more leucine or arginine residues in a multisequence alignment. Only leucine and arginine codons can directly undergo synonymous change at nt1. |
| LRA1nt1nt3    | LRA1nt1 + nt3. |
| noLRA1nt1     | A data subset consisting of all and only those nt1-characters that do NOT encode any leucine or arginine residues in a multisequence alignment. In combination, noLRA1nt1 and LRA1nt1 constitute nt1. |
| noLRA1nt1nt2  | noLRA1nt1 + nt2. In combination, noLRA1nt1nt2 and LRA1nt1nt3 constitute the entire data set, or nt123. |
| nt123_degen1  | A nt123 data set subjected to degen1 coding. |
| nt3_degen1    | A nt3 data set subjected to degen1 coding. |
| LRA1nt1nt3_degen1 | A LRA1nt1nt3 data subset subjected to degen1 coding. |
| nt3_4foldsynon| A data subset consisting of all and only those nt3 characters that are potentially fourfold synonymous, e.g., those encoding glycine and alanine. In nt3_4foldsynon, non-degenerate (i.e., tryptophan and methionine) and potentially twofold- and sixfold-degenerate codons are completely degenerated so as to be uninformative. |
Two other approaches for reducing the effect of synonymous change have recently been developed specifically for higher-level phylogeny. In one approach, all characters in a data matrix are removed that have the possibility of undergoing synonymous change [5], yielding a so-called ndRat1nt2 character set (see Table 1 for definition). The other builds on the original idea of R-T coding at the third codon position [63,44,64,65]. In this approach, which we have previously called deg1 [11], all sites at first and third codon positions that have the potential to undergo synonymous change are individually fully degenerated, yielding a nt123_degen1 character set (see Table 1 for character set definitions).

In the studies carried out to date, deg1 generally supports higher-level nodes as well as or better than all other approaches tested, particularly those that include synonymous change [11,61,62] (Zwick, Regier & Zwickl, in preparation).

The current report is a further exploration of the characters that have been analyzed in Regier et al. (2010) [11] to infer arthropod phylogeny, with the aim of deciding whether their phylogenetic conclusions remain warranted, particularly as regards the newly named groups. The major issues (re)addressed are 1) the phylogenetic signal of the individual genes and the effect of their rates on node support, 2) the amount of data required to achieve strong node support, 3) the effect of character partitioning, and 4) the contribution of synonymous change to node support.

Results

Phylogenetic signal in individual gene segments

A more thorough bootstrap analysis of individual gene segments has been performed than previously [11], but the results remain very similar (Tables S1, S2). For visualization purposes, the number of individual gene segments that support particular nodes with bootstrap values ≥75% is plotted beneath branches on the combined-gene phylogeny (Figure 1). Numerous lower-level and some higher-level groups receive support from multiple individual genes, e.g., up to 43 for Branchiopoda: Anostraca. Notable among the higher-level groups are Arthropoda (4 genes) and Pancrustacea (3 genes). However, many of the nodes along the backbone, including five of the six recently named groups [11] and Symphyla + Pauropoda (see open gray circles in Figure 1), have no individual genes supporting them at that level of the bootstrap. Other higher-level taxonomic groups that have been inconsistently recovered in published studies (i.e., Hexapoda, Oligostraca, Progoneata, Mandibulata) similarly have no individual gene support at that level.

The maximum likelihood tree shown in Figure 1 is based on a combined-gene analysis of the nt123_degen1 data matrix (see Figure 1 in [11]). In that tree, 71 groups out of 78 are recovered by at least three of the four phylogenetic approaches (always including deg1) and all but one of these groups (Oligostraca: Ichthyostega + Mystacocarida) receive >70% bootstrap by at least one of four implemented approaches (groups listed in Table S1). Sixty-three of these have strong combined-gene bootstrap support (i.e., bootstrap ≥80%) by at least one approach, and 46 of the 63 receive single-gene bootstrap support ≥75% from two or more gene segments. The remaining 17 groups, plus all eight that do not have strong combined-gene bootstrap support, receive ≥75% single-gene bootstrap support from one or no gene segments. The latter includes six of the seven nodes of particular interest.

We have defined an approximate metric for the phylogenetic utility of a sequence that takes into account sequence length and number of groups recovered, and that corrects for the variable success rate of amplification and sequencing (see Materials & Methods). This metric has been calculated for each of the 68 gene segments and plotted against its average rate of nonsynonymous change (Figure 2). Over an approximately 10-fold range in average rate, there is a significant (at the level of two standard deviations), but not pronounced, tendency for faster-evolving gene segments to have higher utility. However, there is much scatter, with numerous segments of similar average rates displaying an approximately fourfold difference in utility.

Quantity of data required for strong node support

A random resampling of characters (also called "sites") without replacement from the complete data matrix has been undertaken to estimate the amount of data needed to achieve the observed levels of combined-gene node support (Figure 1; Tables 2, S3), based on the idea that fewer data of the same sort should provide less phylogenetic signal. As a control, a shuffling and reanalysis of the complete data matrix without data reduction results in bootstrap values that vary by ≤2%. However, when the size of the data matrix is reduced to 85%, three nodes (Ammotheidae, Vericrustacea, Entognatha) show a >10% decrease (maximum of 22% for Vericrustacea) in bootstrap support relative to the original data matrix. With a 50% reduction in size for each of five complementary replicated data sets, bootstrap values of 32 nodes decrease by >10% in one or more of the 10 analyses. (Values <50% are treated as = 49% for purposes of computation. Given this, no observations about possible major reductions in bootstrap values are made about nodes having values <60%.) Nodes with bootstrap values in the 60 – 99% range for the original data matrix are preferentially affected by 50% reduction in the data set size. In particular, all 29 nodes in this range show at least one instance of a >10% reduction, while only five of the 32 nodes with 100% bootstrap support show such a reduction (Tables 2, S3). Further emphasizing the abruptness of the boundary, 9 of 11 nodes with bootstrap values from 97 – 99% for the original data set show a ≥30% variation in bootstrap support across the 10 pseudo-replicates, while of the 41 nodes with 100% bootstrap in the original, only one (Oligostraca) shows such a high level of variability. Similar observations hold with further reductions in data set size, i.e., to 33% and 15% of the original matrix (Table S3), although of course even more nodes are now affected.

An additional, intriguing result is that there are seven instances affecting five nodes in which bootstrap values show a >10% increase with data set reduction relative to the original data set, and this is even more striking when one considers that 59 of the 71 nodes already have values ≥90% and, hence, are excluded from the statistic. The complementary, paired bootstrap value for each of the seven instances is always ≥25% less than for the original data set, indicating that phylogenetic signal is not equally distributed across characters and that the most informative characters may be relatively few in number.

Informativeness of subsets of genes with different average nonsynonymous rates

The complete nt123_degen1 data matrix has been partitioned into two equal-sized submatrices consisting of faster and slower evolving gene segments (nonsynonymous changes only), and their maximum likelihood topologies and bootstrap values estimated (Tables 3, S4). Seven nodes are recovered with bootstrap values that are ≥30% higher for the faster genes than the slower genes, while there are 10 nodes for which the slower genes have ≥30% higher bootstrap values than the faster ones. Nodes for which slower genes have higher values tend to have accumulated less overall change (to be more "ancestral") than those for which faster genes have higher values (for visual estimate, see Figure 3; for
quantitative estimate, see inserted table in Figure 3). Although not as decisive, a noLRall1nt2 analysis yields similar results (Table S4).

The findings are similar, although slightly more complicated, when the original nt123_degen1 matrix is partitioned into three equal-sized submatrices -- fastest, medium, and slowest (Tables 3, S4). The fastest and slowest results are largely unchanged from those for the faster and slower submatrices. Additionally however, there are three new nodes for which the medium matrix yields bootstrap values that are ≥30% higher than in the fastest or slowest (both in two of three cases) matrices. Only one of these three nodes receives strong bootstrap support with the complete (nt123_degen1) data matrix, although all do in the medium analysis. Conversely, there are four cases for which the medium matrix yields bootstrap values that are ≥30% lower than in the fastest or slowest matrices. All four of these nodes fail to receive strong support in the medium analysis, but three receive strong support with the complete data set, and the fourth nearly so. Results with noLRall1nt2 data sets yield similar results (Table S4).

Does partitioning by rate lead to strong support for any groups that conflict with those recovered with the complete data set? Indeed, there are four instances of this (Tables 3, S4): 1) 82% bootstrap support for Nymphonidae + Enideidae with the slower genes versus 93% bootstrap support for Ammotheidae + Enideidae (all Pycnogonida) with the complete matrix; 2) 91% bootstrap support for Arthropoda minus Pycnogonida with the slower genes versus 74% bootstrap support for Chelicerata with the complete matrix; 3) 93% bootstrap support for Spirobolida + Polyzoniida with the slower genes versus 67% bootstrap support for Callipodida + Polyzoniida (all Diplopoda) with the complete matrix; and 4) 79% bootstrap support for Chilopoda + Diplapoda with the slower genes versus 67% bootstrap support for Progoneata (all Myriapoda) with the complete matrix.

Figure 2. Phylogenetic utility of 68 gene segments plotted relative to its average rate of nonsynonymous change. As described in greater detail in Materials & Methods, phylogenetic utility (units are displayed on Y axis) is the number of taxonomic groups present in Figure 1 that are recovered with BP ≥75% / nucleotides sequenced for that gene segment × 10^{-3}, corrected for the fraction of missing data. Units for the average rate of nonsynonymous change for each of the 68 gene segments (displayed on X axis) are the number of substitutions at nt2 per site across a 13-taxon tree, corrected for missing taxa (from Table 2 in [5]). The straight line y = bx + c that best fits the data, using linear regression, is shown, together with values for the slope (±1 standard deviation) and the Y-intercept (±1 standard deviation). For convenient cross-reference and identification, individual gene segments are numbered (1 = fastest, 68 = slowest) as in Table 2 of Regier et al., 2008 [5].

doi:10.1371/journal.pone.0023408.g002

Y = 3X + 20.13
slope = 3.0 ± 1.24
Y-intercept = 20.13 ± 1.75
Table 2. Highly variable bootstrap values for selected taxa upon analysis of shuffled, half-sized data matrices.a

| taxonomic group                              | 100% unshuffled | 50% shuffled (1) | 50% shuffled (2) | 50% shuffled (3) | 50% shuffled (4) | 50% shuffled (5) |
|----------------------------------------------|-----------------|------------------|------------------|------------------|------------------|------------------|
| Ammiotheidae + Endeidae                      | 93              | 89 – 83          | 97 – (50)        | 95 – 70          | (50) – 96        | 63 – 95          |
| Ammiotheidae                                | 99              | 100 – 52        | 72 – 98          | 86 – 97          | 94 – 86          | 89 – 96          |
| Tanystylum + Achelia                         | 98              | 58 – 100        | 90 – 96          | 95 – 83          | (50) – 100       | 86 – 96          |
| Chelicerata                                  | 74              | 54 – 78          | 62 – 72          | 69 – 73          | 67 – 62          | (50) – 90        |
| Pulmonata                                    | 65              | 51 – 89          | (50) – 89        | 64 – 86          | 64 – (54)        | (50) – 96        |
| Tetrapulmonata                               | 99              | 100 – (<50)     | 100 – 59         | 99 – 63          | 91 – 95          | 91 – 96          |
| Pleurostigmophora                            | 93              | 91 – 59          | 82 – 82          | 79 – 82          | 77 – 85          | 92 – 67          |
| Scaphopodromorpha + Lithobiomorpha           | 99              | (<50) – 100      | 87 – 99          | 78 – 100         | 96 – 95          | 100 – 82         |
| Diplopoda                                    | 99              | 50 – 100         | 78 – 100         | 74 – 99          | 77 – 99          | 95 – 95          |
| Callipodida + Polyzonida                     | 55              | (<50) – (<50)   | (<50) – (<50)   | (<50) – (<50)   | (<50) – 50      | 86 – (<50)       |
| Symphyla + Pauropoda                         | 92              | (<50) – 73       | 50 – 84          | 56 – 83          | 70 – 64          | (<50) – 90       |
| Oligochaeta                                  | 100             | 95 – 87          | 99 – 55          | 95 – 85          | 84 – 80          | 84 – 90          |
| Alticrustacea                                | 93              | 68 – 75          | 87 – (<50)       | 95 – (<50)       | (<50) – 93      | 60 – 77          |
| Communrostraca                               | 84              | (<50) – 87       | (<50) – (<50)   | 88 – (<50)       | 54 – 82          | (<50) – 87       |
| Eucarida + Peracarida                        | 87              | 90 – (<50)       | 62 – 94          | 58 – 69          | 78 – 68          | 63 – 86          |
| Sessilia                                     | 97              | 82 – 94          | 83 – 93          | 99 – 66          | 81 – 93          | 85 – 86          |
| Milacrustacea                                | 94              | 70 – 58          | 79 – (<50)       | 94 – (<50)       | 73 – 80          | (<50) – 92       |
| Xenocarida                                   | 93              | 75 – 78          | 100 – (<50)      | 94 – (<50)       | 77 – 80          | 52 – 91          |
| Entognatha                                   | 86              | 74 – 73          | 74 – 83          | 63 – 91          | 88 – 65          | (51) – 91        |
| Entomobryomorpha                             | 98              | 88 – 93          | 99 – 67          | 96 – 82          | 69 – 99          | 93 – 91          |
| Pterygotida                                  | 99              | 77 – 95          | 93 – 79          | 96 – 85          | 50 – 89          | 82 – 67          |
| Neoptera                                     | 97              | 73 – 91          | 84 – 75          | 75 – 89          | 92 – 73          | 98 – (<50)       |
| Blattodea + Orthoptera                       | 94              | 86 – 84          | 59 – 98          | 86 – 84          | 99 – (<50)       | 99 – (57)        |

aThis table shows results for only those taxa in the "50% shuffled" analyses that have one or more highly variable (≥30%) bootstrap values. In the five columns at the right (columns 3 – 7) are shown the bootstrap results of five complementary pairs of "50% shuffled" analyses. Complementary results (that is, from different characters within the same bootstrapped data set) are separated by a dash (-). Taxonomic groups not present in the ML topology for that analysis have their bootstrap percentages within parentheses. Results for the complete matrix are shown in the second column for comparison and match those in Figure 1. Complete results for "100% shuffled", "85% shuffled", "50% shuffled", "35% shuffled", and "15% shuffled" are shown in Table S3.

doi:10.1371/journal.pone.0023408.t002

Utility of data partitioning for phylogenetic reconstruction

Partitioning the nt123_degen1 data matrix (without exclusion other than unalignable portions) into two or three linked submatrices that differ in their average rate of nonsynonymous change yields no nodes whose bootstrap values vary by >10% from the unpartitioned nt123_degen1 analysis (Table S4). By contrast, when nt123 is partitioned into two linked submatrices that largely separate synonymous and nonsynonymous change (i.e., noLRall1nt2, LRall1nt3), there is a substantial improvement over the unpartitioned nt123 results. In particular, several nodes in the nt123 partitioned analysis yield bootstrap values that are >10% higher than nt123 unpartitioned, while there are none lower by >1%. Even so, the nt123_degen1, unpartitioned analysis yields 28 nodes that have >10% higher bootstrap values than in the nt123, partitioned analysis, while only one is >10% lower (a generally problematic node at that, namely, Arthropoda minus Pycnogonida).

Testing the informativeness of synonymous signal

The data matrix of Regier et al. (2010) [11] provides a convenient venue for testing the informativeness of synonymous change in supporting ancient divergences. To do this, several approaches have been taken to separate synonymous and nonsynonymous signals, using as a reference the analysis of the nt123_degen1 data matrix, which yields 63 strongly supported nodes (Figure 1). Comparisons of particular note are summarized in Table 4, while the complete trees and their node support values are displayed (Figures 1, 4–8). The noLRall1nt2 data matrix, which largely undergoes only nonsynonymous change, yields 59 strongly supported nodes, and all are in agreement with the nt123_degen1 result (Table 4). The matrix that is complementary to noLRall1nt2, called LRall1nt3 and which is enriched in synonymous change, is approximately equal in size to noLRall1nt2 but recovers only 31 strongly supported nodes that are in agreement with the nt123_degen1 result, plus seven others that strongly conflict (Table 4, Figure 4). Although LRall1nt3 is greatly enriched for synonymous change, some nonsynonymous signal remains. This is directly demonstrated by analysis of the LRall1nt3_degen1 data matrix, in which the number of strongly recovered nodes in agreement with nt123_degen1 increases to 47, while the number of strongly conflicting nodes decreases to one (Table 4, Figure 5).

To further assess the value of synonymous change, the n53 data matrix was analyzed (Table 4, Figure 6). Now, only 19 nodes in agreement with nt123_degen1 are strongly supported, plus six more that strongly conflict. However, this data set too still contains nonsynonymous signal, albeit even less than that in LRall1nt3. To demonstrate this, analysis of the n53_degen1 data matrix, which is almost entirely polymorphic (i.e., almost no A, C, G or T) nevertheless recovers 31 strongly supported nodes in agreement with nt123_degen1, plus only one that strongly conflicts (Table 4, Figure 7).
In order to restrict the analysis almost entirely to synonymous change, all character states at nt3 positions that do not encode a fourfold synonymous codon were fully degenerated (i.e., coded as $N$ making them uninformative), and the resulting nt3_4foldsynon data matrix was analyzed (Table 4, Figure 8). Under these conditions, only 10 nodes in agreement with nt123_degen1 are

Figure 3. Phylogram of higher-level arthropod relationships based on likelihood analysis of aligned, concatenated nt123_degen1 sequences. The cladogenic relationships of 80 "panarthropod" taxa based on analysis of 68 gene segments are the same as those in Figure 1. Nodes with BP that are at least 25% higher with the faster genes than the slower genes are marked with an asterisk. Groups with BP that are at least 25% higher with the slower genes than with the faster genes are marked with a filled square. The shortest distance in number of substitutions / character from the base of a group to a terminal taxon in that group ("minimum distance" in inserted table) was calculated by summing the likelihood branch lengths. Groups are numbered from those with the shortest distance to a terminal (no. 1 = Tanystylum + Achelia) to those with the longest distance (no. 17 = Multicrustacea).

doi:10.1371/journal.pone.0023408.g003
strongly supported, plus one that strongly conflicts. None of these nodes defines a group with more than three terminal taxa, consistent with the hypothesis that synonymous change significantly supports only more recently derived nodes.

A Euclidean distance analysis of the nucleotide composition of $nt3_{4foldsynon}$ shows evidence of strong compositional heterogeneity, when the absolute values of these distances are compared with those in other studies (cf. insert to Figure 8 and [66]). Furthermore, shared bias, as evidenced by the Euclidean tree (Figure 8, insert), correlates with, and is a reasonable explanation of, almost all of the nodes with bootstrap values $\geq 50\%$ that conflict with the $nt123_{degen1}$ likelihood analysis, including:

- 83% bootstrap: *Ammilifer* (Oligostraca: Pentastomida) + *Podura* (Hexapoda: Collembola);
- 66%: *Ammilifer* (Oligostraca: Pentastomida) + *Podura* (Hexapoda: Collembola) + *Heterometrus* (Arachnida: Scorpiones) + *Hadrurus* (Arachnida: Scorpiones);
- 52% bootstrap: *Streptocephalus* (Branchiopoda: Anostraca) + *Amothea* (Pycnogonida); and

| taxonomic group | complete, 1–68 gn, 39261 bp | faster, 1–37 gn, 19842 bp | slower, 38–68 gn, 19419 bp | fastest, 1–24 gn, 13173 bp | middle, 25–43 gn, 12834 bp | slowest, 44–68 gn, 13254 bp |
|-----------------|----------------------------|---------------------------|-----------------------------|---------------------------|----------------------------|-----------------------------|
| 1: Ammotoidea + Endeidae | 93 | 91 | 57 | 99 | 86 | (<50) |
| 2: Nymphonidae + Endeidae | (<50) | (<50) | (<50) | (<50) | (<50) | 82 |
| Ammotheidae | 99 | 79 | 95 | 87 | (<50) | 60 |
| Tanytulus + Achelia | 98 | 97 | 68 | 99 | 81 | (<50) |
| 1: Chelicera | 74 | 73 | 55 | 53 | 96 | (<50) |
| 2: Arthropoda minus Pycnogonida | (<50) | (<50) | (<50) | (<50) | (<50) | 91 |
| Arachnida | 68 | (<50) | 89 | (<50) | (<50) | 83 |
| Pulmonata | 65 | (<50) | 93 | 56 | 57 | 94 |
| Tetrapulmonata | 99 | 99 | 67 | 92 | 76 | 67 |
| Pleurostigmophora | 93 | 94 | (<50) | 95 | (<50) | (<50) |
| Scolopendromorpha + Lithobiomorpha | 99 | 99 | 88 | 89 | 95 | 52 |
| 1: Progoneata | 67 | 64 | (<50) | 74 | (<50) | (<50) |
| 2: Chilopoda + Diplopoda | (<50) | (<50) | 58 | (<50) | (<50) | 79 |
| Diplopoda | 99 | 98 | 83 | 95 | (<50) | 97 |
| 1: Callipodida + Polyzoniida | 55 | (<50) | 72 | (<50) | 99 | (<50) |
| 2: Spirobolida + Polyzoniida | (<50) | (<50) | (<50) | (<50) | (<50) | 93 |
| 3: Callipodida + Spirobolida | (<50) | <50 | (<50) | 55 | (<50) | (<50) |
| Symphyla + Pauropoda | 92 | 59 | 78 | 65 | (<50) | 88 |
| Ostracoda | 60 | (<50) | 85 | (<50) | (<50) | 95 |
| Alticrustacea | 93 | <50 | 94 | (<50) | <50 | 88 |
| Phyllopoidea | 100 | 100 | 100 | 100 | 61 | 100 |
| Multicrustacea | 100 | <50 | 100 | 65 | <50 | 98 |
| Communostraca | 84 | (<50) | 94 | (<50) | (<50) | 95 |
| Sessilia | 97 | 93 | 82 | 97 | (<50) | 87 |
| Miricrustacea | 94 | (<50) | 98 | (<50) | <50 | 91 |
| Xenocarida | 93 | (<50) | 98 | (<50) | 76 | 94 |
| Hexapoda | 100 | 64 | 100 | <50 | 100 | 100 |
| Entognatha | 86 | <50 | 88 | <50 | (<50) | 91 |
| Entomobryomorpha | 98 | 99 | (<50) | 93 | 90 | (<50) |
| Pterygota | 99 | 96 | 71 | 80 | 80 | (<50) |
| Neoptera | 97 | 77 | 96 | (<50) | 91 | 78 |
| Polynoeoptera | 99 | 100 | (<50) | 100 | 96 | (<50) |
| Blattodea + Orthoptera | 94 | 76 | 94 | 86 | (<50) | 86 |

*This table shows results only for those taxa that show sensitivity in their bootstrap values to the rate properties of the underlying data submatrix. All submatrices are fully degenerated ("degen1 coding"). The results for all taxa are shown in Table S3. Results for the complete matrix are shown for comparison in the second column and match those in Figure 1. Columns 3 and 4 show the results of splitting the complete data matrix into two approximately equal-sized subsets (referred to as faster and slower in the text). Column 5–7 show the results of splitting the complete data matrix into three approximately equal-sized subsets (referred to as fast, medium, slowest in the text). Alternative groupings for three taxa in Figure 1, namely, Ammotoidea + Endeidae, Chelicera, Progoneata, and Callipodida + Polyzoniida, are also included because they receive strong support in the tripartite division of the complete data matrix (see text). Bootstrap values for nodes not recovered in the maximum-likelihood topology of a particular analysis are within parentheses. bp, base pair; gn, gene segment.

doi:10.1371/journal.pone.0023408.t003
### Table 4. Comparison of synonymous and nonsynonymous change in terms of node recovery and node support.

| character set      | characters (kbp)* | conflicting nodesb | strongly conflicting nodesc | strongly supporting nodesd |
|--------------------|-------------------|--------------------|-----------------------------|-----------------------------|
| nt123_degen1*      | 39.3              | N.A.               | N.A.                        | (63)                        |
| noLRall1nt2        | 21.8              | 0                  | 0                           | 59                          |
| LRall1nt3          | 17.5              | 18                 | 7                           | 31                          |
| LRall1nt3_degen1*  | 17.5              | 6                  | 1                           | 47                          |
| nt3                | 13.1              | 15                 | 6                           | 19                          |
| nt3_degen1*        | 13.1              | 3                  | 1                           | 31                          |
| nt3_4foldsynon*    | 13.1              | 5                  | 1                           | 10                          |

*The total number of characters in each data set in kilobase pairs (kbp).

**The number of dichotomous nodes with bootstrap values ≥50% in the analysis of the designated character set that fails to match any of the 63 nodes with bootstrap values ≥80% in the analysis of the nt123_degen character set (see Figure 1).

The number of nodes with bootstrap value ≥80% in the likelihood analysis of the designated character set that matches one of the 63 nodes with a bootstrap value ≥80% in the analysis of the nt123_degen character set (see Figure 1).

The number of nodes with bootstrap values ≥80% in the analysis of the designated character set that matches one of the 63 nodes with bootstrap values ≥80% in the analysis of the nt123_degen character set (see Figure 1).

*These four character sets contain numerous polymorphic character states.

doi:10.1371/journal.pone.0023408.t004

- 60%: *Cypridopsis* (Ostracoda: Podocopa) + *Metajapyx* (Hexapoda: Japygidae).

Shared bias in composition may also at least partially explain the level of bootstrap support for many of the presumably correct groupings, including

- 100%: *Heteromurus* + *Hadarus* (both Arachnida: Scorpiones);
- 87%: *Stenochorus* + *Mastigoproctus* + *Phyamus* (all Arachnida: Pedipalpi);
- 100%: *Peripatoides* + *Euperipatoides* (both Onychophora: Peripatopsidae);
- 97%: *Peripatoides* + *Euperipatoides* + *Peripatus* (all Onychophora);
- 100%: *Limulus* + *Carcinoscorpius* (both Xiphosura);
- 100%: *Pulex* + *Mastichoides* (both Hexapoda: Archaeognatha);
- 99%: *Acanthocyclops* + *Mesocyclus* (both Copepoda: Cyclopoida); and
- 67%: *Oithalamus* + *Semibalanus* (both Thecostraca: Sessilia).

### Testing the informativeness of indels

An inspection was undertaken of a slightly realigned arthropod data matrix [see Dataset S1 in SUPPORTING INFORMATION] for introns whose evolutionary history appeared to be highly constrained. The results are summarized in Table 5 (see Discussion).

### Discussion

#### The challenges of higher-level arthropod systematics

Morphology and gene-based studies of higher-level arthropod phylogeny have been ongoing for more than 75 and 20 years, respectively [67–69], stimulated by increasing evolutionary and paleontological knowledge of arthropods and their huge radiation at the species level, and also by numerous major findings of developmental biologists (summarized in [70,71]). However, agreement on many higher-level relationships remains problematic, even with spectacular improvements in methodologies (e.g., polymerase chain reaction and pyrosequencing) and phylogenetic theory (e.g., better likelihood models and faster algorithms). Recently, however, Regier et al., 2010 [11] provided strong evidence for many higher-level arthropod relationships outside Chelicerata, based on analysis of 62 protein-coding nuclear genes (shown in modified form in Figure 1). The current report is a further analysis of their data matrix and results in order to probe whether or not that report represents a major advance in our knowledge of arthropod phylogeny, an overinterpreted and failed report, or something in-between.

An intriguing aspect of Figure 1 is that almost all nodes, excepting a few within Chelicerata, receive strong bootstrap support. Such strong support is oftentimes interpreted as providing a good indication that there is a strong signal supporting those relationships [72], and larger data sets should on average have stronger signal. Of course, decisiveness does not necessarily translate to accuracy [18], but then what might be the source of such strong signal other than phylogeny? First however, we emphasize that the bootstrap percentage is a conservative metric relative to the posterior probability metric generated in Bayesian analyses [73,74]. This is often obvious in studies that show support values from maximum likelihood and Bayesian analyses for a common topology, e.g., the analytical results for the data set in Regier et al., 2010 [11]. Given the still unresolved controversy over potentially inflated posterior probabilities [75], we feel that support for phylogenies based on Bayesian posterior probabilities alone warrants substantial skepticism and, therefore, was not used in this report. However, we note that a Bayesian analysis of the nt123_degen1 data set yielded posterior probabilities of 1.0 for all nodes within Mandibulata except one (see Supplementary Figure 3 in [11]).

Compositional heterogeneity is a possible explanation for high support values and is known to be a widespread cause of phylogenetic inaccuracy (e.g., [36–51, but see 52]). In the present study, however, this is highly unlikely to be the explanation for the very widespread strong support because three of the four favored analytical approaches eliminate the largest source of the problem, namely, synonymous change, and the fourth, namely, implementation of the codon model, functionally “downweights” synonymous change due to its relatively rapid evolution [11].

Another potential non-phylogenetic source could be problematic modeling of evolutionary change. However, model testing was
performed [76], and the four favored analytical approaches use three different types / classes of models (nucleotide, codon, amino acid models), yet still recover nearly identical topologies [11]. Furthermore, partitioning the nonsynonymous data, which are all of the same sort, namely, rather conservative, protein-coding nuclear gene sequences, yielded no major improvement (Table S4).

While other artifacts remain possible, e.g., [77,78], if the relationships shown in Figure 1 are generally accurate, then why have many of the higher-level relationships not previously been strongly supported? The current study demonstrates that insufficient quantity of data is likely to be one of the main factors. Of the 68 gene segments tested, none individually support with bootstrap values \( \geq 75\% \) deep nodes like Mandibulata, Oligostraca, Hexapoda, Symphyla + Pauropoda, or five of the six newly named pancrustacean groups, even though all are supported in the combined-gene analysis with bootstrap values \( \geq 80\% \). A reasonable interpretation is that their strong support in the combined-gene analyses results from the cumulative effect of small amounts of phylogenetic signal from multiple genes. Earlier studies were restricted to fewer genes and taxa than in Regier et al., 2010 [11], so it may not be surprising that these groups were not strongly supported. Hexapoda deserves special comment, since there has been controversy as to its monophyly (e.g., see [79]). Our results are quite definitive on this matter. Hexapoda is strongly supported as monophyletic, although it is a "difficult" node because no single gene yields a bootstrap value \( \geq 75\% \). Clear support for hexapod monophyly is similarly difficult to find in morphological analyses, despite its widespread acceptance. We also note that hexapod monophyly can be strongly supported based on analysis of nt123, but, at least for the current gene set, this high level of support is largely the result of the unusually biased nucleotide composition at nt123 and LRall1 that positively reinforces phylogenetic signal [5].

An alternative interpretation for the paucity of strong support at the single-gene level is that some or all of the genes contribute substantial conflicting signal that cancels the phylogenetic signal, keeping individual node support low and raising doubts about the ultimate source of the combined-gene support. But this seems unlikely to be a predominant explanation because individually the gene segments mostly strongly support groups present in Figure 1 (Table S1). And, for the few cases in which conflicting groups are supported, this support typically stems from only a single gene fragment (Table S2). Further, little conflict is apparent when randomly subsampled matrices that are 15% – 85% of the full data set in size are analyzed. The most significant one is Arthropoda minus Pycnogonida (77% bootstrap) that conflicts with Chelicerata (74% bootstrap).

Submatrices corresponding to different rate categories of genes similarly do not provide striking evidence for major internal conflict. Only four groups conflict with ones in the complete matrix (Pycnogonida: Nymphoniidae + Endeididae versus Pycnogonida: Ammotheidae + Endeididae; Arthropoda minus Pycnogonida versus Chelicerata; Diplopoda: Spirobolida + Polyzyoniida versus Diplopoda: Callipodida + Polyzyoniida; and Chilopoda + Diplopoda versus Progonata). And only the first one yields strong support for both (conflicting) alternatives. More importantly for this report, none of these groups conflict with any of the six newly named groups, Symphyla + Pauropoda, Mandibulata, Oligostraca, or Hexapoda.

Although not a formal optimality criterion, consistency of results across different analytical approaches is also quite striking in Regier et al., 2010 [11]. Two approaches, called degen1 and noLRall1nt2, analyze in a likelihood framework those nucleotides that undergo mostly nonsynonymous change. A related approach -- likelihood analysis of amino acid change -- is conceptually similar, but of course the underlying model is very different. The fourth approach -- likelihood analysis of codon change -- is different in that codons, not single nucleotides, are analyzed and no data, including those that undergo synonymous change, are excluded from the analysis. Importantly, all four methods largely recover the same groups, including Symphyla + Pauropoda, Mandibulata, Oligostraca, Hexapoda, and the six newly named pancrustacean groups.

So, is the vast majority of conclusions in Regier et al., 2010 [11] credible? Beyond the philosophical point that all scientific conclusions are defeasible, it is clear that nodes recovered with the complete data matrix with <100% bootstrap support are more sensitive to data subsampling than those with 100% support. Furthermore, the definition of 80% bootstrap support as "strong" is a heuristic device only; it does not refer to some innate or even broadly accepted category. Therefore, it seems likely that strongly supported nodes in the range of 80 - 99% bootstrap can still be sensitive to changes in the amount of data. Had the amount of data been less, the probability of recovering that group with strong support would have been reduced (Tables 2, S3). By contrast, many fewer of the nodes with 100% bootstrap are sensitive to data subsampling at the levels tested in this report. Even when the data set is reduced to 15% of the complete matrix, there are still 23 of 52 nodes that receive 100% bootstrap support, although none correspond to the deep nodes Symphyla + Pauropoda, Mandibulata, Oligostraca, Hexapoda, and the six newly named pancrustacean groups. What this suggests is that, had fewer data been generated and analyzed in Regier et al, 2010 [11], it is likely that at least some of these high-interest groups would not have been strongly supported. Therefore, given that their strong recovery is near the limit of an admittedly arbitrary designation of "strong", it would still be valuable to generate even more data as confirmation. This is despite the lack of even modest support for alternatives to these high-interest groups, unlike the more problematic situation with Chelicerata and the placement of Pycnogonida [80,81].

Nonsynonymous rates and information content

While analyses of individual gene segments fail to recover most deep-level nodes with bootstrap values \( \geq 75\% \), including five of the six newly named groups [11] plus Symphyla + Pauropoda, Mandibulata, Oligostraca, and Hexapoda, nevertheless, many other nodes are supported at this level (Figure 1). The total number of individual gene segments that recover particular nodes varies dramatically (from 0 to 43, see Table S1). In an effort to capture an assessment of individual gene utility, a statistic was devised that expresses the total number of nodes supported by a single gene segment after adjusting for differing sequence lengths and amount of missing data, and this was expressed relative to the gene segments' average rates of nonsynonymous change (Figure 2; other metrics are
Nuclear Genes for Higher-Level Arthropod Phylogeny

COLOR GUIDE:
- Hexapoda
- Xenocarida
- Vericrustacea
- Oligostraca
- Myriapoda
- Chelicerata
- outgroup

LRall1nt3_degen1 ML + BP

# : BP, node in nt123_degen1
# : BP, node not in nt123_degen1
The utility of data degeneration, data exclusion, and partitioning by rate on phylogenetic accuracy

Elsewhere, we have justified the utility of excluding synonymous change in our deg1 and noLRall1nt2 analyses when analyzing deep-level arthropod relationships [61,62] (Zwick, Regier & Zwick, in preparation). A comparison of results from the analysis of nt123 and nt123_degen1 in this report reinforces this point (Table S4). Bootstrap support for 31 nodes (out of 71 analyzed) is lower by >10% points with nt123 than with nt123_degen1. By contrast, only three nodes are >10% higher with nt123. One (Chilopoda + Diplopoda) is likely to be incorrect, based on consistent, but modestly supported, recovery of Progoneata (Figure 1; Figure 1 in [11]). Another (Arthropoda minus Pycnogonida) is questionable but possible [80], given the striking instance of its receiving strong deg1 support (bootstrap 91% for the slowest genes, Table S4), plus the generally modest deg1 support for Chelicera, although the medium genes recover Chelicera with bootstrap 96% (Table S4). And the third (Pulmonata) is possibly correct based on its consistent and modest-to-strong deg1 support (Tables 3, S4; Figure 1; Figure 1 in [11]), but remains in conflict with the current hypotheses based on morphology [84].

As an alternative to data degeneration (e.g., nt123_degen1) and exclusion (e.g., noLRallnt2), we have tried partitioning nt123 into mostly nonsynonymous and mostly synonymous change (noLRallnt2 + LRallnt3), and this results in a modest improvement relative to nt123, unpartitioned (Table 4). Now, only 27 nodes show >10% lower bootstrap support relative to nt123_degen1. However, the take-home message even with this further analysis remains unchanged: synonymous change hinders deep-level phylogenetic analysis (see also discussion below).

Restricting the analysis largely to nonsynonymous change through degeneration, we have asked whether partitioning the entire data set by the average rates of evolution for the different gene fragments, followed by their separate modeling, could provide a benefit. The answer is that, whether split into two or three rate categories, there are no nodes whose bootstrap values differ by >10% from nt123_degen1, unpartitioned. Even so, it is also clear that the separate rate categories of genes, as well as individual gene segments, do have distinct signals and can provide dramatically different levels of support for distinct nodes. Together, these two observations suggest that, in the current case with the complete nt123_degen1 data set, applying a single model to the entire data set is about as good as using multiple, independent models.

As just mentioned, an important finding of the current study is that individual gene segments and separate rate categories of genes are informative at different phylogenetic levels. In particular, more slowly evolving genes (only nonsynonymous changes considered) tend to support nodes that are closer to the backbone on the phylogram shown in Figure 3, matching the conventional wisdom that slow genes are better for supporting deeper nodes [19,20,5,21–24]. Conversely, faster genes provide more support for nodes that are more recently derived.

The (non)utility of synonymous change for inferring deep-level arthropod phylogeny

As we have documented in this report, synonymous change, much more so than nonsynonymous change, can be misinformative of the correct phylogeny (Table 4). While this is often times attributed to its more rapid evolution, that in itself is unlikely to be an adequate explanation, since, for example, likelihood methods do not require equal rates of change across the character matrix in order to make accurate inferences. Rather, there must be a bias introduced by synonymous change that might then be compounded by faster evolution. Nucleotide compositional heterogeneity is one such bias, and it has been well documented that this is a more
The dominant feature of characters undergoing synonymous than nonsynonymous change [25–28,5]. The likelihood models that we have used assume a single nucleotide composition, biased or not, across the entire data set. While there is increasing interest in modeling compositional heterogeneity, it is still a work in progress [53–56].

Figure 6. Phylogram derived from likelihood analysis of the nt3 character coding of the 68 gene segments. Bootstrap values ≥50% are displayed above internal branches. All and only those bootstrap percentages that correspond to nodes not present in the nt123_degen1 topology (Figure 1) are in italics, boldface, and brown in color. Notice that the branch lengths for the degenerated data matrix nt3_degen1 (Figure 6) are much shorter than in this figure. Units are substitutions / character.

doi:10.1371/journal.pone.0023408.g006
one justification for our alternative approach of using nucleotide degeneration or exclusion. However, there is still an outstanding question, which is, how informative of deep-level arthropod phylogeny is synonymous change?

To test the informativeness of synonymous change, we have generated data sets that are progressively more enriched in their potential for synonymous change (Table 4; Figures 4–8; cf. Figure 1). What we observe is that the number of nodes which are strongly supported decreases as the fraction of synonymous change increases, and that strongly supported incorrect nodes are almost entirely due to synonymous change. For example, while it has been stated that nt3 characters should not be removed because...
Nuclear Genes for Higher-Level Arthropod Phylogeny

COLOR GUIDE:
Hexapoda
Xenocarida
Vericrustacea
Oligostraca
Myriapoda
Chelicerata
outgroup

nt3_4foldsyon ML + BP
# : BP, node in nt123_degen1
# : BP, node not in nt123_degen1

nt3_4foldsyon Euclidean distance

0.05

0.5

Miliesium
Lepas
Chthamalus
Semibalanus
Loxothylacus

Polyzonium
Limnadia
Triops
Acanthocylops
Mesocylops
Thulinius
Cypriopsis
Metajapyx
Cydia
Antheraea

Polyxenus
Narceus
Euryupalopus

Lynceus
Libinia
Cryptocellus
Amblyomma
Derocheilocaris
Cranerostigmus
Hutchinsoniella

Scutigerella

Streptocephalus
Amphithea

Daphnia
Skogsbergia
Neogonodactylus
Lithobius
Idiogarypops
Abacion
Hansenella
Periplaneta
Ctenolepsis

Pedetontus
Machioides
Speleonectes

Argulus
Orchesella
Tomocerus
Prokoxenienia

Eurytemora
Eumesocampa
Libellula
Ischnura

Heterometrus
Hadrurus
Armillifer
Podura
Mastigoproctus
Phrynus
Stenochirus
Harbansus
Armadillosmum
Aphonopelma
Peripatoides
Euperipatoides
Peripatus
Limulus
Carcinoscorpius
Eremocosta
Sculopendra
Scutigera
Tanystylum
Artemia

Dinothrombium

August 2011 | Volume 6 | Issue 8 | e23408
they contribute useful phylogenetic information [29–33], in the current case nt3 strongly supports only 19 of 63 nodes strongly supported by nt123_degen1, plus 15 conflicting nodes (6 strongly conflicting), while nt3_degen1, which is almost entirely nonsynonymous and polymorphic, still strongly supports 31 of 63 nodes with only 3 conflicting nodes (1 strongly conflicting). When only nt3 characters of fourfold synonymous codons (nt3.fieldsynon) are analyzed, thereby largely eliminating the potential for non-synonymous change, the number of strongly supported nodes drops to 10 out of 63 plus five conflicting nodes (1 strongly conflicting). Even this relatively small level of support is suspect, however, when coupled with information about nucleotide heterogeneity (Figure 8). In particular, the large majority of groups for which there is bootstrap support ≥50% have shared, strongly biased compositions, whether those taxonomic groups are conflicting or not. While this doesn’t prove that there isn’t a sequence-based signal derived from synonymous change, it demonstrates that compositional heterogeneity is likely to factor into the combined result. Furthermore, all groups supported by synonymous change are restricted to just a few taxa. Clearly, synonymous change is not informative of deeper-level relationships in this study. In this case, a premise that synonymous change ought to be informative and should be retained would be based on the mistaken impression that change at nt3 is entirely synonymous, when in fact, the change at nt3 that is informative of deep-level arthropod phylogeny is largely or entirely non-synonymous. In contrast, the often-mentioned nt12 analyses retain synonymous change at nt1 and discard useful non-synonymous information at nt3, both of which presumably can result in lowered node support relative to nt123_degen1 (e.g., see Supp. Figure 2 in [11]; Figure 1 in [5]).

Analysis of indels

In this study “high-quality” (defined in Materials & Methods) indels that grouped two or more taxa were investigated as a source of phylogenetic information, and 19 were identified (Table 5). Eleven indels support eight groups that are already well supported in sequence-based analyses, e.g., all receive ≥75% bootstrap support from 3 – 42 individual genes. Seven additional indels support seven additional groups that are clearly incorrect. Thus, indels analysis did not prove useful (e.g., see also [85]), although more complex modeling of indel evolution would likely prove useful, as it has for nucleotide and amino acid evolution.

Conclusion

The current results, plus another to appear shortly (Zwick, Regier, and Zwickl, in preparation), provide additional support and explication of the phylogenetic results presented in Regier et al. (2010) [11]. In particular, there is additional justification for the major emphasis on four analytical approaches, three of which emphasize non-synonymous change under differing assumptions and a fourth which directly models codon change. This is because synonymous change provides little, if any, useful phylogenetic signal for the deeper nodes, while contributing substantial misinformative signal, perhaps mostly attributable to composition-

| Taxonomic groups recovered | Number of indels |
|---------------------------|------------------|
| Hexapoda: Collembola     | 2                |
| Hexapoda: Odonata        | 2                |
| Hexapoda: Lepidoptera    | 1                |
| Pancrustacea: copepoda   | 1                |
| Pancrustacea: Branchiopoda: Diplostraca | 2 |
| Pancrustacea: copepoda: Cyclopoida | 1 |
| Pancrustacea: Malacostraca | 1 |
| Arthropoda / Onychophora + Tardigrada | 1 |
| Pycnogonida / Arachnida: Pseudoscorpiones | 1 |
| Hexapoda: Collembola: Tomoceridae + Hexapoda: Collembola: Poduridae | 1 |
| Pancrustacea: ostracoda: Podocopa + Tardigrada: Parachel a | 1 |
| Hexapoda: Ephemeroptera + Hexapoda: Diplura | 1 |
| Pancrustacea: oligostraca + Arachnida: Opilliones | 1 |
| Pancrustacea: copepoda + ostracoda: Myodocopa | 1 |
| Arachnida: Tetrapulmonata + Arachnida: Pseudoscorpiones | 1 |

*High quality* indels are those that have relatively unambiguous alignments – no staggered ends and identical lengths but not necessarily identical sequences (see Materials & Methods).

*Present in Figure 1 and considered a valid taxonomic group.

*Not present in Figure 1 and unlikely to be a valid taxonomic group.

doi:10.1371/journal.pone.0023408.t005

---

**Figure 8.** Phylogram and Euclidean distances derived from analysis of the nt3.fieldsynon character coding. In the main figure, bootstrap values ≥50% are displayed above internal branches of the phylogram based on likelihood analysis of the 68 gene segments. All and only those bootstrap percentages that correspond to nodes not present in the nt123_degen1 topology (Figure 1) are in italics, boldface, and brown in color. Units for the phylogram are substitutions / character. In the smaller, inserted figure are shown Euclidean distances based solely on nucleotide composition. Symbols (+, V, O, Δ) identify the same clusters of taxa in both the ML topology and the Euclidean distance diagram for convenient cross-reference. Units for the Euclidean distance diagram are per cent. doi:10.1371/journal.pone.0023408.g008
al heterogeneity. The current report clarifies why strong node support for higher-level relationships has been so challenging, namely, that single genes contribute insufficient signal for robust support, necessitating the combined analysis of multiple genes. In fact, the current report suggests that the up-to-approximately 40 kilobase pairs / taxon in the data matrix of Regier et al. (2010) [11], while providing "strong" support for 10 "high-interest" nodes that previously had not been strongly and consistently supported (i.e., Allocreastacea, Communostraca, Hexapoda, Mandibulata, Mirarcrustacea, Multicrustacea, Oligostraca, Vericrustacea, Xenoconica, Synphyla + Pauropoda), nevertheless proved sensitive to modest reductions in the amount of data. Thus, a reasonable question would be to inquire into the consequence of modest-to-large increases in the amount of data. While we detected not even modest support for alternative resolutions of the "high interest" nodes under various data set manipulations, the outcome of increasing the total amount data remains an important experiment to do. Thus, it will be necessary to reinvestigate these relationships with even more data, as will inevitably occur in this new age of relatively cost-efficient phylogenomics.

Materials and Methods

Taxon and gene sampling

Taxon and gene sampling are identical to that reported in Regier et al., 2010 [11]. That report includes a complete listing of taxa (75 species Arthropoda, 3 species Onychophora, 2 species Tardigrada) and their higher classification (Supplementary Table 1 in [11]), as well as GenBank numbers for sequences of the 68 gene fragments (Supplementary Table 4 in [11, Table S5 in this report]). In that earlier report [11], as well as [5], one taxon within Pycnogonida (Colossendeis sp., lab code name "Col") has now been shown to have been misidentified (see Acknowledgments). It has been reidentified as Nymphon sp. (Pycnogonida: Nymphoniidae). The lab code name remains "Col". The original misidentification changes nothing as regards conclusions in those earlier publications about the position of Pycnogonida within Arthropoda. In the current report, we use "Nymphon sp." and "Nymphoniidae" throughout.

Data sets

All data submatrices were derived from one of two master matrices that include all taxa and all gene fragments, either as is (nt123) or after fully degenerating nucleotides (nt123_degen1) using IUPAC ambiguity codes at those sites that can potentially undergo synonymous change, thereby making synonymous change largely invisible and reducing the effect of compositional heterogeneity but leaving the inference of nonsynonymous change largely intact (summarized in Table 1). For example, in nt123_degen1 CAC and CAT (His) are both coded CAY, while TTA, TTG, CTT, CTC, CTA, and CTG (Leu) are all coded YTN. The nt123_degen1 data matrix and the nt123 data matrix can both be downloaded as Supplementary Data of Regier et al., 2010 [11]. Software to degenerate sequences is available at http://www.phylotools.com. A character-exclusion mask (2313 characters out of 41,574 total characters) of ambiguously aligned nucleotide characters was also invoked, and is identical to that in Regier et al., 2010 [11]. Using the degen1 approach, one set of submatrices, called single-gene matrices, was derived from each of the 68 gene fragments. Another set consisted of gene fragments grouped by their average rate of nonsynonymous change (according to Table 2 in [5]), such that there were two approximately equal-sized nt123_degen1 submatrices (faster and slower; 19842 and 19419 characters, respectively, and corresponding to gene fragment numbers 1–37 and 39–68 in Table 2 of [11]), and another such that there were three approximately equal-sized nt123_degen1 submatrices (fastest, medium, slowest; 13173, 12834, and 13254 characters, respectively, and corresponding to gene fragment numbers 1–24, 25–43, and 44–68 in Table 2 of [11]).

These same collections of gene fragments, namely, 1–37 / 38–68 and 1–24 / 25–43 / 44–68, as well as all genes combined (1–68) were also analyzed in a distinct manner from degen1 coding but still with the aim of restricting the analysis to nonsynonymous change. Previously, we have called this alternative the ndRoll1nt2 approach, and it is based on character exclusion for nt123 (non-degenerate) rather than nucleotide degeneration [5] (http://www.phylotools.com). With this approach, two complementary submatrices, called ndRoll1nt2 and LRall1nt3, are defined as follows: The ndRoll1nt2 character subset consists of all characters at the second codon position (nt2) plus only those nt1 characters that encode no leucine or arginine codons (noLR). The LRall1nt3 character subset consists of all characters at the third codon position (nt3) plus any and all nt1 characters not present in ndRoll1nt2. Since leucine and arginine codons are the only ones that undergo synonymous change at nt1, their absence from ndRoll1nt2 leads to a dramatic reduction in the amount of synonymous change inferred over a tree. Software to create ndRoll1nt2 and LRall1nt3 character set definitions is available at http://www.phylotools.com. In this set of analyses, the noLR analysis of gene fragments 1–68 and of the five subsets with differing rates occurred with ndRoll1nt2 only, after excluding LRall1nt3.

To explore the informativeness of synonymous change, progressively more restrictive data submatrices were constructed and analyzed with and without degeneration. LRall1nt3 has already been described, and it was compared with LRall1nt3_degen1. Analysis of the latter captures all of the nonsynonymous change in LRall1nt3 with very little or no synonymous change. Another pair of submatrices was nt3 and nt3_degen1. The final submatrix, called nt3_degen1, included only those sites at nt3 that encode fourfold-degenerate codons, e.g., alanine and glycine; all other sites were converted to N. Analysis of the nt3_degen1 submatrix should capture entirely synonymous change. Degeneration of nt3_degen1 would yield all Ns and hence was not performed.

For another set of degen1 submatrices, character order for the entire nt123_degen1 data matrix was first randomized using a Perl script (available at http://www.phylotools.com), which randomly resampled all the characters in the matrix without replacement. This reordering (or shuffling) was repeated in series 10,000 times to ensure randomness. Submatrices of differing sizes were constructed (100% as a control, 85%, 50%, 33%, 15%). Only one submatrix each of 100%, 85%, and 15% of the complete size was constructed. For the 50% matrices, five independently randomized 100% matrices were split into five pairs of complementary matrices (ten 50% matrices total). Likewise, two independently randomized 100% matrices were each split into five pairs of complementary matrices (ten 50% matrices total).

Further, two types of character partitioning were undertaken for total data (nt123). In one, the complete data matrix (nt123, non-degenerate) was split into two complementary subsets, namely, ndRoll1nt2 and LRall1nt3, in order to separate a major source of character variation, namely, nonsynonymous and synonymous change. In the phylogenetic analysis, GTR + G + I models were applied to each subset with unlinked parameters. In another partitioning scheme, the nt123_degen1 matrix was analyzed by applying GTR + G + I models with unlinked parameters to three subsets described above, namely, fastest, medium, slowest.
Insertion / deletion (indel) events were analyzed for phylogenetic informativeness, but only "high-quality" indels, which consisted of those that have relatively unambiguous alignments, defined as those with no staggered ends and identical lengths but not necessarily identical sequences. Before doing this, however, a slightly improved manual alignment relative to that in Regier et al. (2010) [11] was undertaken, this time using a MAFFT alignment ([96]; default values with the BLOSUM62 scoring matrix) of amino acids to guide our decision-making about realignments within the nucleotide data set. Relative to the original alignment [11], there were adjustments in the positioning of sequences for 12 species, 9 of which resulted in realignments of <10 characters, one of 22 characters, one of 63 characters, and one of 93 characters. In addition, we have discovered 592 base pairs of incorrect sequence in the mayfly Hexagenia limbata (codename: May) that was duplicated in silico from another species’ RNA polymerase II (largest subunit) sequence, and this has now been replaced with Ns. The realigned, non-degenerate data matrix used for indel analysis (nt123) is included in Supporting Information as Dataset S1. Separately, the degenerate, realigned data matrix (nt123, deg1) is available at http://www.phylotools.com) was reanalyzed under likelihood using GARLI 1.0. There were no changes in the maximum likelihood topology relative to Regier et al., 2010 [11], and bootstrap support for all nodes changed by ≤4%.

Phylogenetic analysis

All phylogenetic analyses are based on the maximum likelihood criterion applied to nucleotides, as implemented in GARLI (v. 0.961), v. 1.0 with and without a character-partitioning feature added; ([87]), using the GTR + G + I model. All other parameters are default values. We used the program default settings, including random stepwise addition starting trees, except that we halved the number of successive generation passes yielding no improvement in likelihood score that prompts termination (genthresh=10000), as suggested in the GARLI manual. The number of replicate searches used to obtain a maximum likelihood tree estimate for each data set ranged from 550 – 674, following Regier et al. (2009) [66]. The number of pseudo-replicates of the non-parametric bootstrap analyses varied, depending on results being compared and the computational requirements but always matched or exceeded the targeted number of pseudo-replicates. For the data partitioning results, the number of bootstrap replicates is 520 – 599. For the randomized-sequence comparisons, the number of bootstrap replicates is 470 – 668. However, in some cases, the computational time was simply too long to perform as large a large number of replicates as for the aforementioned analyses. Thus, rather than keep all analyses at an identical low minimum, we chose instead to accept larger differences between separate analyses for the benefit of additional accuracy for the majorities of them. Hence, for the single-gene analyses, the number of bootstrap replicates ranges from 209 to 666. For testing the informativeness of synonymous signal, the number of bootstrap replicates is 651 – 673, except for the computationally intensive nt3_4foldson analysis, which was 236. The tree shown in Figure 1 is taken directly from Regier et al. [11], and it is based on 1065 bootstrap replicates. All of these numbers are such that the statistical variation around the mean is still only a few percentage points. Other than the single-gene bootstrap analyses, the number of heuristic search replicates per bootstrap replicate varied from 1–5. For the single-gene bootstrap analyses only, we increased the number of search replicates / bootstrap replicate to 50, except for the longest genes, ef2 (40 search reps) and polii (30 search reps), from 1 previously presented in Supplementary Table 3 of Regier et al. (2010) [11], in order to provide a more accurate estimate for each bootstrap pseudoreplicate. However, the resulting changes in bootstrap percentages were relatively minor (Table S1,S2).

Optimal-tree searches and bootstrap analyses were parallelized using Grid computing [99] through the Lattice Project [99]. For purposes of comparison only, we oftentimes collectively refer to nodes with bootstrap values ≥75% in our single-gene studies, and to bootstrap values ≥80% in our multigene studies. In the combined-gene analyses only, we define for heuristic purposes only bootstrap values ≥80% as "strong."

Estimating phylogenetic utility of individual gene segments

To provide a quantitative estimate for how useful a particular gene segment is for phylogeny reconstruction, an approximate metric called "phylogenetic utility" was developed. Based on single-gene likelihood bootstrap analyses, the total number of taxonomic groups present in Figure 1 that are recovered by the single gene fragment with bootstrap ≥75% is divided by the length (in nt ×10^-3) of the fragment, and then corrected for missing taxa by multiplying this fraction by (80 taxa / (80 taxa – number of missing taxa)). The calculated value of phylogenetic utility for each segment was plotted relative to its rate of nonsynonymous change (see Table 2 in [5]). Linear regression was performed using Linest, a spreadsheet function available in OpenOffice (http://www.openoffice.org). Two features of this metric are worth pointing out. First, it estimates the total number of nodes that could be recovered, but in most systematic studies not all nodes are of equal interest. Second, this metric estimates utility as information density (i.e., total information / fragment length), but in most systematic studies what matters in a practical sense is the total amount of information in a sequenceable gene fragment.

Compositional heterogeneity and synonymous change

To describe compositional heterogeneity in a data matrix, we calculated Euclidean distances on the proportions of the four nucleotide frequencies treated as independent characters using a Perl script (available at http://www.phylotools.com). A separate script available from the same web site was used to restrict the total data matrix to fourfold degenerate codons, which were subsequently split by codon position to produce a matrix (called nt3, 4foldson) of those nt3 characters that are encoded by fourfold degenerate codons.

Supporting Information

**Table S1** Single-gene bootstrap values (≥75% only, nt123, deg1) for taxonomic groups (nodes) present in Figure 1. This table lists bootstrap values for taxa identified in Figure 1 based on analysis of single genes. (XLS)

**Table S2** Single-gene bootstrap values (≥75% only, nt123, deg1) for taxonomic groups NOT present in Figure 1. This table lists bootstrap values for taxa not recovered in the analysis shown in Figure 1 based on analysis of single genes. (XLS)

**Table S3** Bootstrap values based on analysis of shuffled data matrices of varying sizes (100% to 15% of complete data matrix). This table lists bootstrap values after randomizing character order in the 100% data matrix and splitting it into portions of varying sizes for analysis (100% to 15% of complete data matrix) without replacement. A subset of the Table-S3 results are also shown in Table 2. This Table-2 subset includes results only for those
taxonomic groups that show particularly highly variable bootstrap values between replicates of the 50% matrices.

(DOC)

Table S4 Bootstrap values based on analysis of data sets and subsets differing in their average rates of nonsynonymous change. The complete data set is split into two or three subsets based on average rates of nonsynonymous change of individual genes, and bootstrap analyses are performed to estimate the informativeness of the different rate category ranges. A subset of the Table-S4 results is also shown in Table 3. This Table-3 subset includes results only for those taxonomic groups that show particularly highly variable bootstrap values from differing rate category ranges of gene segments.

(DOC)

Table S5 GenBank accession numbers (also cited in [11]).

(DOC)

Dataset S1 A Nexus-formatted data set that includes nucleotide sequence data (n476) for 80 taxa and 62 genes, slightly realigned relative to that in Regier et al., 2010 [11] (see Materials and Methods). The species codenames used in this realigned data set are also identified by their complete genus-species names in Table SM1 of Regier et al., 2010 [11].

(NE)

Acknowledgments

We especially thank Jeffrey W. Shultz for comments early on in this study, as well as Clif Cunningham and all other co-authors listed in Regier et al., 2010 [11], who inspired the current report; Michael Cummings and Adam Banzet for valuable assistance with grid computing through The Lattice Project; Derrick Zwickl for providing a version of GARLI prior to its official release that could analyze partitioned data sets; and Florian Leese for bringing to our attention the misidentification of a pycnogonid species used in this and earlier studies (Colossendeis sp. now identified as Nymphon sp., [90]; see Material and Methods).

Author Contributions

Conceived and designed the experiments: AZ JCR. Performed the experiments: JCR AZ. Analyzed the data: JCR AZ. Contributed reagents/materials/analysis tools: JCR AZ. Wrote the paper: JCR AZ.

References

1. Strausfeld NJ (1998) Crustacean - Insect relationships: The use of brain characters to derive phylogeny amongst segmented invertebrates. Brain Behav Evol 52: 106–206.
2. Fuenzalida M, Harosch S, Wagele JW (2004) The brain of the Remipedia (Crustacea) and an alternative hypothesis on their phylogenetic relationships. Proc Natl Acad Sci U S A 101: 3868–3873.
3. Mallatt J, Giribet G (2006) Further use of nearly complete 28S and 18S rRNA genes to classify Ecdysozoa: 37 more arthropods and a kinorhynch. Mol Phylogenet Evol 40: 772–794.
4. Dunn CW, Hejnol A, Matus DQ, Pang K, Browne WE, et al. (2008) Broad phylogenomic sampling improves resolution of the animal tree of life. Nature 452: 745–750.
5. Regier JC, Shultz JW, Ganley ARD, Hussey A, Shi D, et al. (2008) Resolving arthropod phylogeny: Exploring phylogenetic signal within 41 kb of protein-coding nuclear gene sequence. Syst Biol 57: 920–930.
6. Aleshin VV, Mikhailov KV, Konstantinova AV, Nikitin MA, Rusin LY, et al. (2008) On the phylogenetic position of insects in the Pancrustacea clad. Molecular Biology 43: 804–818.
7. Ertas B, von Reumont BM, Wagele JW, Misol B, Burmester T (2009) Hemocyanin suggests a close relationship of Remipedia and Hemadapta. Mol Biol Evol 26: 2711–2718.
8. Roeding F, Borner J, Kube M, Klages S, Reinhardt R, Burmester T (2009) A 454 sequencing approach for large scale phylogenomic analysis of the common benthic foraminiferan genus Rhabdoschwelleria. Mol Phylogenet Evol 53: 826–834.
9. Koemenen N, Jenner RA, Hoemen M, Stemme T, von Reumont BM (2010) Arthropod phylogeny revisited, with a focus on crustacen relationships. Arthropod Structure & Development 39: 88–110.
10. Meusemann K, von Reumont BM, Simon S, Roeding F, Strausso S, et al. (2010) A phylogenomic approach to resolve the arthropod tree of life. Mol Biol Evol 27: 2451–2464.
11. Regier JC, Shultz JW, Zwick A, Hussey A, Ball B, et al. (2010) Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. Nature 463: 1079–1083.
12. Baurain D, Brinkmann H, Philippe H (2006) Lack of resolution in animal phylogeny amongst closely related arthropod lineages. Mol Phylogenet Evol 40: 659–664.
13. Peck KS, Philippe H, Schreiber F, Epenbeck D, Jackson DJ, et al. (2010) Improved phylogenetic taxonomic sampling noticeably affects multi-gene phylogenetic inferences: The relationship between red algae and green plants as a case study. Mol Biol Evol 26: 2511–2524.
14. Plass LC, Perkins SL (2008) Nodalization and base composition bias explain phylogenetic conflict in Phialidiales. Genomics 9: 435–442.
15. Yang Z (1994) On the best evolutionary rate for phylogenetic analysis. Syst Biol 43: 55–65.
16. Kallersjo M, Albert WA, Farris JS (1999) Homoplasy increases phylogenetic structure. Cladistics 15: 91–93.
17. Simmons MP, Carr TG, Neill K (2004) Relative character-state space, amount of potential phylogenetic information, and heterogeneity of nucleotide and amino acid characters. Mol Phylogenet Evol 32: 913–926.
18. Brinkmann H, Philippe H (1999) Improved phylogenetic mapping: Closely spaced cladogeneses or undetected systematic errors? Mol Phylogenet Evol 26: 1171–1178.
19. Smith MJ, Neill K (2004) Identifying and removing fast-evolving sites using compatibility analysis: An example from the Arthropoda. Syst Biol 53: 978–989.
20. Hampl V, Hug L, Leigh JW, Dacks JB, Lang BF, et al. (2009) Phylogenomic analyses support the monophyly of Eucaryota and resolve relationships among eukaryotic supergroups. Proc Natl Acad Sci U S A 106: 3859–3864.
21. Inagaki Y, Nakajima Y, Sato M, Sakaguchi M, Hashimoto T (2009) Gene sampling can bias multi-gene phylogenetic inferences: The relationship between red algae and green plants as a case study. Mol Biol Evol 26: 1171–1178.
22. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
23. Pratt RC, Gibb GC, Morgan-Richards M, Phillips MJ, Hendy MD, et al. (2009) Toward resolving deep neovars phylogeny: Data, signal enhancement, and priors. Mol Biol Evol 26: 313–326.
24. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
25. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
26. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
27. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
28. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
29. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
30. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
31. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
32. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
33. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
34. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
35. Baurain D, Brinkmann H, Philippe H (2006) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc R Soc Lond B 267: 1213–1221.
39. Tarrío R, Rodríguez-Trelles F, Ayala FJ (2000) Tree rooting with outgroups when they differ in their nucleotide composition from the ingroup: The Drosophila saltans and saltator groups, a case study. Mol Phylogenet Evol 16: 344–349.

40. Tarrío R, Rodríguez-Trelles F, Ayala FJ (2001) Shared nucleotide composition biases among species and their impact on phylogenetic reconstructions of the Drosophilidae. Mol Biol Evol 18: 1464–1473.

41. Rosenberg MS, Kumar S (2003) Heterogeneity of nucleotide frequencies among evolutionary lineages and phylogenetic inference. Mol Biol Evol 20: 610–621.

42. Jermiin LS, Ho SYW, Alasbari F, Robinson J, Larkum AWD (2004) The biasing effect of compositional heterogeneity on phylogenetic estimates may be underestimated. Syst Biol 53: 630–643.

43. Naylor GJ, Brown WM (1998) Amphioxus mitochondrial DNA, chordate phylogeny, and the limits of inference based on comparisons of sequences. Syst Biol 47: 61–76.

44. Phillips MJ, Penny D (2002) The root of the mammalian tree inferred from nonstationary model of optimal outgroups in phylogenetics. Syst Biol 54: 493–500.

45. Huelsenbeck, JT, Degnan PH, Wemmerge JF (2005) Nonhomogeneous model of sequence evolution indicates independent origins of primary endosymbionts within the Enterobacteriales (beta-Proteobacteria). Mol Biol Evol 22: 530–532.

46. Cos CJ, Foster PG, Hirt RP, Harris SR, Embley TM (2000) The archaeabacterial origin of eukaryotes. Proc Natl Acad Sci U S A 103: 20356–20361.

47. Hardman J, Braun EL, Braum J, Huddleston CJ, Bowie RC, et al. (2008) Phylogenetic evidence for multiple losses of flight in ratite birds. Proc Natl Acad Sci U S A 105: 13462–13467.

48. Rota-Stabelli O, Telford MJ (2008) A multi criterion approach for the selection of optimal outgroups in phylogeny: Recovering some support for Mandibulata over Myriochelata using mitogenomics. Mol Phylogenet Evol 48: 103–111.

49. Whelan S (2008) Spatial and temporal heterogeneity in nucleotide sequence evolution. Mol Biol Evol 25: 1683–1694.

50. Song H, Sheffield NC, Cameron SL, Miller KB, Whiting MF (2010) When phylogenetic assumptions are violated: Based compositional heterogeneity and among-site rate variation in beetle mitochondrial phylogenomics. Syst Ent 35: 429–448.

51. Conant GC, Lewis PO (2001) Effects of nucleotide composition bias on the success of the parsimony criterion in phylogenetic inference. Mol Biol Evol 18: 1024–1033.

52. Lockhart PJ, Steel MA, Hendy MD, Penny D (1994) Recovering evolutionary trees under a more realistic model of sequence evolution. Mol Biol Evol 1: 605–612.

53. Foster, PG (2004) Modeling compositional heterogeneity. Syst Biol 53: 485–495.

54. Jayaswal V, Robinson J, Jermiin L (2007) Estimation of phylogeny and invariable sites under the general Markov model of nucleotide sequence evolution. Syst Biol 56: 155–162.

55. Sheffield NC, Song H, Cameron S L, Whiting M F (2009) Nonstationary evolution and compositional heterogeneity in beetle mitochondrial phylogenomics. Syst Ent 35: 429–448.

56. Naylor GJ, Brown WM (1998) Amphioxus mitochondrial DNA, chordate phylogeny, and the limits of inference based on comparisons of sequences. Syst Biol 47: 61–76.

57. Phillips MJ, Penny D (2002) The root of the mammalian tree inferred from nonstationary model of optimal outgroups in phylogenetics. Syst Biol 54: 493–500.

58. Huelsenbeck, JT, Degnan PH, Wemmerge JF (2005) Nonhomogeneous model of sequence evolution indicates independent origins of primary endosymbionts within the Enterobacteriales (beta-Proteobacteria). Mol Biol Evol 22: 530–532.

59. Cos CJ, Foster PG, Hirt RP, Harris SR, Embley TM (2000) The archaeabacterial origin of eukaryotes. Proc Natl Acad Sci U S A 103: 20356–20361.

60. Hardman J, Braun EL, Braum J, Huddleston CJ, Bowie RC, et al. (2008) Phylogenetic evidence for multiple losses of flight in ratite birds. Proc Natl Acad Sci U S A 105: 13462–13467.

61. Rota-Stabelli O, Telford MJ (2008) A multi criterion approach for the selection of optimal outgroups in phylogeny: Recovering some support for Mandibulata over Myriochelata using mitogenomics. Mol Phylogenet Evol 48: 103–111.

62. Whelan S (2008) Spatial and temporal heterogeneity in nucleotide sequence evolution. Mol Biol Evol 25: 1683–1694.

63. Song H, Sheffield NC, Cameron SL, Miller KB, Whiting MF (2010) When phylogenetic assumptions are violated: Based compositional heterogeneity and among-site rate variation in beetle mitochondrial phylogenomics. Syst Ent 35: 429–448.

64. Conant GC, Lewis PO (2001) Effects of nucleotide composition bias on the success of the parsimony criterion in phylogenetic inference. Mol Biol Evol 18: 1024–1033.