Whole-Proteome Tree of Arthropods: An “alignment-free” phylogeny of proteome “books”

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Methodology article

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Abstract

Background: An “organism tree” of a group of extant-organisms can be considered as a conceptual tree to capture a simplified narrative of the evolutionary course among the organisms. Due to the difficulties of whole-genome sequencing for many organisms, the most common approach has been to construct a “gene tree” by selecting a group of genes common among the organisms, “align” each gene family and estimate evolutionary distances. Despite broad acceptance of the gene trees as the surrogates for the organism trees, there are important limitations and confounding issues with the approach. During last decades, whole-genome sequences of many extant-arthropods became available, providing an opportunity to construct a “whole-proteome tree” of the arthropods, the largest and most species-diverse group of all living animals.

Results: An “alignment-free” whole-proteome tree of the arthropods shows that (a) the demographic grouping-pattern is similar to those in the gene trees, but there are notable differences in the branching orders of the groups and the sisterhood relationships between pairs of the groups; and (b) almost all the “founders” of the groups have *emerged* in an “explosive burst” near the root of the tree.

Conclusion: Since the *whole*-proteome sequence of an organism can be considered as a “book” of amino-acid alphabets, a tree of the books can be constructed, without alignment of sequences, using a text analysis method of Information Theory, which allows comparing the information content of whole-proteomes. Such tree provides another view-point to consider in telling the narrative of kinship among the arthropods.

Background

For several decades, due to the technical difficulties of whole genome sequencing, the most practically feasible and common approach to construct an evolutionary tree of a group of extant organisms has been to construct a “gene tree”, where a group of genes common among the organisms are selected, “align” highly homologous sequence-regions for each gene family, and quantify the divergence of certain characteristics, e.g. point substitution rates, to estimate evolutionary distances among the genes. Such “alignment-based” gene trees may represent the phylogeny of the selected genes, and may reveal, depending on the selected genes, various aspects, but not full aspects of the “organism tree”, because the aligned regions of the selected genes account, in general, for a very small fraction of the genome coding for *all* genes. Furthermore, this approach makes a set of *assumptions* about evolutionary process such as: (a) the collection of such highly homologous sequence-regions contains enough information to represent each organism and (b) one type of mutation, e.g., point substitutions, within the aligned regions account for the most important mutational events of evolution. In addition, there are several other limitations and confounding issues with the approach [1, 2]. Strictly speaking, such gene trees may reveal the evolution of the selected genes, but not of the organisms [1]. “Alignment-free” approach [2, 3] applied to whole-proteome sequence can reduce some of the limitations and the confounding issues of the alignment-based approach.

Recent availability of a large number of whole-genome sequences prompted us to consider whether an alternative approach, such as alignment-free methods applied to whole-proteome, may provide information about the similarities and differences between the two types of trees, since the basic assumptions used are very different. In alignment-free method, whole-genome information can be used under the assumptions that: (a) all or most of whole genome sequence of an organism represents the organism and (b) a combination of *all types of mutations*, such as point substitution, insertion/deletion of various length, recombination, duplication, transfer or swapping of gene, domain or motif, etc., contribute to the evolution of the organism. At present, complete or near complete sequences of many arthropods (over 134 species as of 2020) of broad diversity are available in a public database [4], which allows one to construct a whole-proteome tree of arthropods (ToAr).

We have constructed a whole-proteome ToAr, which turned out to be more stable *topologically* than those of whole genome or whole transcriptome ToAr (see Supplementary Information Fig. S1), constructed using Information-Theory based Feature Frequency Profile method [5], an “alignment-free” method, on the extant arthropods. Here we describe the main features of the ToAr on grouping and kinship among the groups.

Results

To compare the gene trees with the whole-proteome tree of arthropods we chose two recent and very comprehensively-analyzed gene trees: The first one is the recent “alignment-based” gene tree of 144 insect taxa based on 1,478 single-copy protein-coding nuclear genes [6], where the tree was “inferred through a maximum-likelihood analysis of 413,459 amino acid sites divided into multiple metapartitions, branch lengths were optimized and node ages estimated from 1,050,000 trees sampled from trees separately generated for 105 partitions that included all taxa”. The authors also suggest the relationship between the geological origin of insect clades and major geological and biological events in chronological time scale. The second is the gene tree for 76 arthropod taxa [7], where the tree was “inferred from 569 to 4,097 single copy protein-coding genes among the six multi-species orders, crustaceans, and non-spider chelicerates and 150 single copy genes for the orders represented by a single species and deeper nodes”. Both gene trees agree on the branching order of the Order groups, and on similar time of the emergence of the founders of the groups in chronological time scale.

In comparing our whole-proteome tree to these two gene trees, we focus on two aspects: grouping patterns and phylogeny of the groups. For the former, we “cluster” our study population by several *unsupervised* clustering algorithms using *only* the evolutionary distances estimated from the “divergence” distances among whole-proteome characters (see **Construction of whole-proteome Tree of Arthropods in Materials and Methods**) with no assumption of specific evolutionary models; we then ask whether the “clustering pattern” is similar to the “clading pattern” in the gene trees and in our whole-proteome tree. For the phylogeny of the groups, we compare the order of branching of the groups and their emergence on the evolutionary progression scale (see “**Cumulative Genomic Divergence(CGD)**” in

“Evolutionary Progression Scale” in Materials and Methods), which are derived from the evolutionary distance estimation *and* specific evolutionary models assumed.

A. Demographic grouping by clustering and clading

Clustering: We have tested several unsupervised clustering algorithms, such as Principal Component Analysis (PCA), Multi-Dimensional Scaling (MDS), and t-Distributed Stochastic Neighbor Embedding (t-SNE) [8, 9], all of which are based only on the evolutionary distances, estimated by the divergence of whole-proteome sequences among all pairs of the arthropods (see **Construction of whole-proteome Tree of Arthropods in Materials and Methods**). Of these, t-SNE and classical PCA showed the clustering pattern most compatible to the current grouping of arthropods based on common or scientific names. Figure 1, a t-SNE clustering, shows about 17 clusters of the extant arthropods in this study that are most stable under a wide range of “perplexity”, the adjustable parameter of t-SNE, which is roughly related to the “looseness” of each cluster. Another clustering by PCA (see Supplemental information, Fig. S1) also shows about similar number of clusters, although some of them are not as well resolved as in t-SNE. This is not expected, because the assumptions and algorithms in t-SNE and PCA are completely different.

Clading: Figure 2 shows the topology of whole-proteome ToAr, constructed using neighbor-joining method implemented in BIONJ [10, 11], where we use the divergence distances of whole-proteome sequences as the estimates for the evolutionary distances (see **Construction of whole-proteome Tree of Arthropods in Materials and Methods**), combined with an evolutionary model of maximum parsimony (minimum evolution) in a way that the “correct neighbors” to be joined are those that minimize the total sum of branch-lengths at each stage of step-wise joining of neighbors starting from a star-like tree [10]. The tree shows that all 17 clusters in Fig.1 can be identified among the clades in the ToAr.

Robustness of grouping: Furthermore, the grouping pattern by clustering *and* clading based on whole-proteome information also agrees well with those of the gene trees except for Hemiptera group (see **Comparison with recent gene trees in Discussions**).

Thus, it is surprising that the demographic grouping pattern is robust regardless not only of the information type (morphology, select gene-characters or whole proteome characters), but also of the methods (clustering or clading) used in grouping the extant organisms. However, not surprisingly, there are significant differences from the gene trees in branch-length and branching order of the groups, which are used to predict the position, in the evolutionary progression scale, at which the “founders” of each group emerged (see below).

B. Emergence of the “Founders” of all major groups

For the following results we define “Cumulative Genomic Divergence (CGD)” for an internal node of the ToAr as the cumulative scaled-branch-length from the tree root to the node (see **“Cumulative Genomic Divergence(CGD)” in “Evolutionary Progression Scale” in Materials and Methods**) to represent the extent

of “Evolutionary Progression”, where the progression is scaled such that the root of ToAr is set at CGD=0 (see **Outgroup** in **Discussions**) and the leaf positions of the extant organisms at CGD near 100.

“Arthropodal burst” near the root of ToAr: Figures 3 shows the whole-proteome ToAr with CGD values. It reveals that the “founders” (for definition, see Supplemental information, Fig. S3) of all major groups (at Subphyla and Order levels) emerged in a burst within a short evolutionary progression span between CGD of 1.6 and 5.8 (marked by a small red arc), near the root of the tree in Figs. 3, 4 and 5. This burst is reminiscent of the “deep burst” of the founders of all kingdoms of Life in the whole-proteome tree of Life [12].

Order of emergence of the “founders” for all major groups: Figures 4 and 5 show a series of staged emergence of the founders of all major groups within the burst. The first groups to emerge (branch out) in a narrow range of CGD values of 1.6 and 1.9 are the founders of Subphylum Crustacea and Chelicerata (arachnids and Atlantic horseshoe crab). The founders of the next groups to emerge are Hemiptera-A group (aphids and a psyllid) at CGD of 3.7, followed by the founders of Diptera group at CGD of 4.1. The founders of the remaining five Order-level groups (Lepidoptera, Hemiptera-B (bugs, a planthopper and a whitefly), Coleoptera, Blattodea + a thrips, and Hymenoptera groups) emerged at CGD of 4.4, 4.8, 5.2, 5.8, and 5.8, respectively.

Discussions

Similarities in grouping patterns

It is surprising that the grouping patterns at Order level between the gene trees [6, 7] and our whole proteome tree are very similar (see below for one notable exception of Hemiptera) despite the facts that the types of input data (multiply aligned regions of selected genes vs. whole proteome) and grouping methods (clustering vs. clading) are different. A possible implication is that each group members evolved, during the most of the evolutionary progression, largely “isolated” within the group without genomic intermixing between the groups, thus, resulting in each group having much smaller genomic variation within the group than between the groups. This interpretation is consistent with our whole-proteome tree, where the founders of all Order groups emerged in a burst near the root of the tree, followed by a long gradual evolution spanning the most of the evolutionary progression scale.

Dissimilarities in branching orders and branch-lengths: It is not surprising that the branching orders and branch-lengths are not similar, because the assumptions under which the estimations for evolutionary distances among the organisms are very different: in the gene trees, the distances are calculated for the aligned portions of the selected genes for, e.g., point-substitutional mutations, while in our tree they are done for all proteins in the whole proteome for all types of mutations.

Evolutionary progression scale vs. chronological time scale

“Chronological time scale” is linear, but the “evolutionary progression scale” is most likely non-linear, because any catastrophic events on earth may accelerate, decelerate or diversify the evolutionary progression. However, the direction of arrows in both scales are the same, suggesting that the two scales need to be calibrated when sufficient fossils and other independent record become available (see **“Cumulative Genomic Divergence (CGD)” in “Evolutionary Progression Scale” in Materials and Methods**). Meanwhile, we use the evolutionary progression scale to compare the order of emergence of the founders of various major groups under the assumption that the scale can be calculated by the whole proteomic information divergence.

Emergence of the founders of major groups: While cognizant of the difference and similarity of the two scales, the most dramatic difference is observed in the span of the scales when the founders of all major groups at Order level emerged. In the gene trees, the founders of all the groups at Order level emerged gradually during a long chronological time of about 350 Myrs corresponding roughly 60% of about 570 Myrs between the tree root to the extant arthropods (Fig. 1 of reference 6) or about 210 Myrs corresponding to roughly 37% of the same full chronological scale (Fig. 2 of reference 7). In drastic contrast, the founders of all the major groups in our tree emerged within about 4% of the full evolutionary progression scale in a sudden burst (“Arthropodal burst”: see Figs. 3 and 4) near the root of the ToAr. This observation has an important evolutionary implication in constructing the narrative for the birth of the arthropods.

On notable differences in grouping and phylogenic positions

As mentioned earlier all grouping and most of the order of emergence of the groups at Order and Subphylum levels agree between the two types of trees except some notable differences in Hemiptera and Blattodea as discussed below. These differences may get resolved once the whole genome sequences of more diverse members of the two groups become available.

Hemiptera

In the gene trees [6, 7], the bugs, cicadas, and aphids form Hemiptera clade. But, in our ToAr as well as in t-SNE (Fig. 1) and PCA clustering plots (Supplemental Information, Fig. S1), Hemiptera is divided into two separate clades, which we call Hemiptera-A (aphids and a psyllid) and Hemiptera-B (planthopper, whitefly, stink bug, bed bug, cat flea, ash borer). The former is consisting of two of the most “primitive” Hemiptera feeding passively on mostly one plant species or its closely related plants, is at the basal position of *all* Hexapoda and the latter is at basal or sister position to a larger clade consisting of Coleoptera, Blattodea and Hymenoptera (see Figs. 3 and 5 for their phylogeny and Fig. 1 for clustering). Our clustering results (which are based solely on the whole proteome sequences, and not constrained by (a) the assumptions and related confounding issues associated with algorithms of tree building under given evolutionary models or (b) any or combination of particular phenotypes, such as metamorphosis, eusociality, or passive/active defensive survival modes etc.), show that the difference in the *whole-proteome information contents* between the two clades is greater than those within each clade.

Blattodea

Two termites (Blattodea) and one thrips (Thysanoptera), both eusocial and hemimetabolous, form a clade in our ToAr and the clade is sister (or basal) to Hymenoptera group, which is also eusocial but holometabolous (see Fig. 5). However, in the gene trees, Blattodea (cockroaches and termites, which are eusocial and hemimetabolous), as a member of a larger clade Polyneoptera [6] or as a separate clade [7], is basal or sister to all other Order groups of Insecta, which are mostly non-social and hemi- or holometabolous [7]. This is in contrast to what we observe in our ToAr, where non-social Hemiptera-A, is the basal group of all Hexapoda, consistent with the notion that eusociality of Blattodea is likely to be a trait acquired late in arthropod evolution.

Outgroup

As was described in our earlier work on whole-proteome trees [12, 13, 14] we constructed the proteome sequence of an “artificial (faux) arthropod” to use as an outgroup member of a ToAr by “shuffling” [15, 16] the alphabets of the whole proteome sequence of an organism in the study group. We used two such artificial arthropods (named R28612 and r12957) to form the outgroup for the study. Each has the same size and amino acid composition of an extant arthropod, but does not have information to life.

Conclusions

A whole-proteome tree constructed using “alignment-free” approach is based on a set of assumptions and methods that are fundamentally different from those used in the most commonly accepted “alignment-based” gene trees. Our tree provides another view-point to consider in telling the narrative of kinship among the arthropods. Some of the significant differences between the two types of trees of extant arthropods may have important implications in constructing a narrative of the evolutionary kinship among arthropods. Our tree raises the following debatable issues:

1. The founders of *all* order-level groups of arthropods emerged in a “burst” rather than through a long stretch of chronological time as suggested in gene trees;
2. Hemiptera-A, consisting of some of the most “primitive” Hemiptera, may be the basal group of all members of Insecta, rather than eusocial Blattodea as suggested in gene trees;
3. Blattodea may be sister to Hymenoptera, a eusocial group.

Methods

Aim: Construct a “whole-proteome tree” to provide another view-point of building a narrative for the evolution of the extant arthropods.

Design: 1) To treat each whole proteome-sequence of an arthropod as a “book” of the amino acid alphabets; 2) To compare the information content among all books of the extant arthropods for which whole genome sequences are available by calculating the divergence of the information content using a

text comparison method of Information Theory [17, 18, 19]; 3) To construct the whole proteome tree of the arthropods to estimate a cumulative genomic divergence as the evolutionary distance for each internal node of the tree; 4) To compare the whole proteome tree with recent gene trees [6, 7] in grouping and the kinship among the groups.

Sources and selection of proteome sequences

We downloaded the proteome sequences for 134 arthropods from NCBI RefSeq DB using NCBI FTP depository [4; as of December 2019]. Protein sequences derived from all organelles were excluded from this study. Also excluded from our study are those derived from whole genome sequences assembled with “low” completeness based on two criteria: (a) the genome assembly level indicated by NCBI as “contig” or lower (i.e. we selected those with the assembly levels of ‘scaffold’, ‘chromosome’ or ‘complete genome’), and (b) the proteome size smaller than the smallest proteome size among highly assembled arthropod genomes (*Anopheles gambiae str. PEST* with 14,089 proteins at “chromosome” assembly level; TaxID 180454).

All taxonomic names and their taxon identifiers (TaxIDs) of the organisms in this study are from NCBI taxonomy database, and listed in Supplementary Information, Dataset S1, where "N/A" indicates an unassigned taxonomic order.

Construction of whole-proteome Tree of Arthropods

Based on our earlier experiments of constructing whole-proteome trees of prokaryotes [13], fungi [14] and all life forms [12] by Feature Frequency Profile (FFP) method, following choices have been made to obtain a topologically stable whole proteome ToAr of maximum parsimony (minimum evolution) by BIONJ [10, 11]: a) Among three types of genomic information (DNA sequence of the whole genome, RNA sequence of whole transcriptome and amino acid sequence of whole proteome) whole-proteome trees are most “topologically stable” as estimated by Robinson-Foulds metric [20] at respective “optimal Feature-length” (see Supplementary Information, Fig. S2 ; b) For FFP as the “descriptor” of the whole proteome of each organism, the optimal Feature-length is about 10 amino-acid string (see also Supplementary Information, Fig. S2); and 3) Jensen-Shannon Divergence (JSD) [17] is an appropriate measure of “divergence of information content” or distance of dissimilarity between two whole-proteome descriptors for constructing the distance matrix of BIONJ. It is important to note that such FFP of a whole-proteome sequence of an organism has all the information necessary to reconstruct the original whole proteome sequence.

“Cumulative Genomic Divergence (CGD)” and “Evolutionary progression scale”

In Information Theory, the Jensen-Shannon Divergence (JSD), bound between zero and one, is commonly used as a measure of the dissimilarity between two probability distribution of informational features. The FFP as the descriptor for a linear sequence information of the whole proteome of an organism is such a probability distribution. Thus, a JSD value of two FFPs, used as a measure of the divergent

distance between two proteome sequences, is also bound between 0 and 1, corresponding to the JSD value between two FFPs of identical whole proteome sequences and two completely different whole proteome sequences, respectively. (For easy visibility, we multiply the branch-lengths in iTOL [21] by 200). Any whole proteome-sequence “dissimilarity” between two extant organisms accumulated during the evolution can be considered as caused by changes of, ultimately, genomic sequences due to point substitutions, indels, inversion, recombination, loss/gain of genes, etc. as well as other unknown mechanisms, and they will bring JSD somewhere between 0.0 and 1.0 depending on the degree of the sequence divergence.

In this study the collection of the JSDs for all pairs of the study organisms plus 2 out-group members (see **Outgroup** in **Discussions**) constitutes the “distance matrix” for BIONJ [10, 11]. Since all the branch-lengths are derived from the JSD values, the cumulative branch-length of an internal node, which we call “cumulative genomic divergence (CGD)” of the node along the presumed evolutionary lineage, can be considered as the point of evolutionary stage reached by the node on an “evolutionary progression scale”. For convenience of assigning the nodes on the progression scale, CGDs are scaled such that the CGD value at the root node of ToAr is set to zero and the leaf nodes of the extant organisms to come out around 100, corresponding to the fully evolved genomic states of the organisms, which we define as the end point of the “evolutionary progression scale” for the organisms at present (see Fig. 3).

Clustering methods

We use two unsupervised methods to observe the clustering patterns based solely on whole proteome sequences: Principal Component Analysis (PCA) and t-Distributed Stochastic Neighbor Embedding (t-SNE) [8, 9]. Both are dimensional reduction methods, but with strength and weakness for our purposes. which help to visualize any clustering pattern in the data distribution. Both are based only on the evolutionary distances, estimated by the divergence of whole-proteome sequences among all pairs of the study arthropods. In PCA, the distances within a cluster as well as between two clusters are quantitative, thus, two close clusters nearby may not resolve well. In t-SNE, which applies Machine Learning to emphasize the resolution of nearby clusters, but the inter-cluster distances are ignored, thus, not quantitative.

Declarations

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Author contribution: Conceptual design of the study and speculative interpretations and implications of the results by SHK; filtering and curation of genomic and proteomic sequence data from NCBI database, computational-algorithm design, programming and execution by JJC; interpretation of computational results by SHK and JJC; unsupervised clustering by various algorithms were performed by BJK; manuscript preparation by SHK with extensive discussions with JJC and BJK; all figures are designed by SHK and JJC.

Computer code availability: The FFP programs for this study (2v.3.0) written in GCC(g++) is available in Github: <https://github.com/jaejinchoi/FFP>.

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Figures

Figure 1

Unsupervised clustering (grouping) of 134 extant arthropods by t-SNE. t-SNE [9] is a machine learning algorithm of clustering a high-dimensional data into a two or three dimensional space by emphasizing resolution of clusters, but de-emphasizing the distances between the clusters. There are about 17 clusters with their common names in parentheses. These clusters can be assorted into six Order groups and two Subphylum groups (in colored-bold letters), correspond to Hemiptera, Lepidoptera, Diptera, Coleoptera, Blattodea, Hymenoptera, Chelicerata and Crustacea.

Figure 2

Topology of the linear representation of whole-proteome ToAr The colors of the first colored-band distinguish organisms in different Classes, and those of the second among different Orders (the names of different color-bands are shown in Fig. 3). Scientific names and common names, when available, of each organism are also listed. The silhouettes of sampled organisms are shown next to their names. To emphasize the clading pattern, all branch-lengths are ignored. The first two items refer to two members of

the outgroup (see Outgroup in Discussions) constructed by shuffling [15, 16] the whole-proteome sequences of two arthropods. The visualization of the ToAr was made using iTOL [21].

Figure 3

Cumulative branch-lengths of the “pie” representation of whole-proteome ToAr. This view of the tree shows all branch-length to emphasizes the progression of evolution of each member in the study from the origin at CGD=0 to the extant forms of the arthropods at CGD= near100. The small red arc near the root is at CGD=5.8, by which point of the evolutionary progression, the founders of “all major groups” (consisting of 7 Order groups and 2 Subphylum groups shown in Fig. 1) have emerged, suggesting that the remaining 94.2 on CGD scale corresponds to further diversification of the founders within each major group toward their extant forms. The visualization of the ToAr was made using iTOL [21].

Figure 4

Expanded view of Fig. 3 near the root of the whole-proteome ToAr Examples of the founders of all major groups are shown as blue dots, and the groups within two major groups, Diptera and Hymenoptera, as red dots. The visualization of the ToAr was made using iTOL [21].

Figure 5

Simplified whole-proteome ToAr On the left is cumulative genomic divergence values, which ranges from zero to around 100, and they correspond to the extent of evolutionary progression from the root of the ToAr to the extant leaves. For simplicity, “singletons” (that do not belong to any named groups) are not shown and all the leaf nodes and their branches of a common-named group (in parenthesis) are combined into a single dotted line coming out from their common ancestor (internal) node shown as a sphere. Each internal node represents a “pool of founding ancestors” (see Supplementary Information Fig. S3), and each circle represents an internal node containing all members of a clade with known name (in parenthesis). Dotted vertical lines are to indicate that they are arbitrarily shortened to accommodate large jumps of CGD values within a limited space of the figure. The double-headed arrow at bottom right indicates the short range of the CGD values, within which the founders of all the major groups of the extant arthropods in the study have emerged. For our interpretation of horizontal lines and vertical lines, see Supplementary Information Fig. S3.

Supplementary Files

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- [Dataset1Arthropodacopy.xlsx](#)
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