Phylogenetic Tree Construction Using K-Mer Forest-Based Distance Calculation

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Abstract—Phylogenetics is one of the dominant data engineering research disciplines based on biological information. More particularly here, we consider raw DNA sequences and do comparative analysis in order to come up with meaningful conclusions. When representing evolutionary relationships among different organisms in a concise manner, the phylogenetic tree helps significantly. When constructing phylogenetic trees, the elementary step is to calculate the genetic distance among species. Alignment-based sequencing and alignment-free sequencing are the two leading distance computation methods that are used to find genetic relatedness of different species. In this paper, we propose a novel alignment-free, pairwise, distance calculation method based on k-mers and a state of art machine learning-based phylogenetic tree construction mechanism. With the proposed approach, we can convert longer DNA sequences into compendious k-mer forests which gear up the efficiency of comparison. Later we construct the phylogenetic tree based on calculated distances with the help of an algorithm build upon k-medoid clustering, which guaranteed significant efficiency and accuracy compared to traditional phylogenetic tree construction methods.

Keywords—Phylogenetics; genetic relatedness; genetic distance; k-mer forest; k-medoid clustering.

1 Introduction

Species are different from each other based on inherited characteristics. This differentiation can happen in numerous ways. When measuring such differences between species, genetic distance can be identified as one of the critical criteria which can be used to extricate them[1]. Discern mosquito vectors of malaria, filling the unclear positions in taxonomy evolution, growing drug resistance over time to deduce treatments for diseases [2] are some of the real-world use cases where genetic distances are applicable. As mentioned in the “Simons Genome Diversity Project”, some pairs of ancestors of the present human population, say African and Non-African, were extensively separated a long time ago when considering genetic distances [3]. Moreover, genetic
distances are used to identify the origins of biodiversity. Knowledge of biodiversity is essential for breeding domestic and phylogenetically valued animals. Some rare breeds should be protected to keep the genetic diversity equilibrium in the future [4].

The phylogenetic tree (Evolutionary tree) is a branching diagram that shows the evolutionary relationships among various organisms. It is constructed by considering the similarities and differences of species genetically or physically. Phylogenetic trees can be used in scenarios such as identifying the origins of species, finding common ancestors, cluster species etc. Genetic distance calculation is the crucial step of phylogenetic tree construction. The proposed two-stage methodology in this paper is introduced as a more accurate and efficient way of constructing phylogenetic trees. In the first stage, a novel genetic distance computation method is used to build a distance matrix. In the next step, the sophisticated machine learning approach is used to construct the tree based on the distance matrix. Other than tree construction, the distance matrix is one of the key inputs of most of the biological applications, including population genetics and metagenomic binning. Similarly, phylogenetic trees are also used in various bioinformatics based researches as a fundamental component. Thus, from the distance matrix construction and phylogenetic tree construction methods we made here an open path to several research areas branching in the field of bioinformatics

2 Background

There are two stages in the pipeline of phylogenetic tree construction. In the first stage, it is required to get all to all genetic distances among species. The final output of this stage is to build the distance matrix. In the next step, phylogenetic tree construction is happening based on this distance matrix. Genome sequence alignment [5] is one approach used to compute genetic distances in bioinformatics which rely on alignment. BLAST [6] a well-known Basic Local Alignment Search Tool, can be taken as an example of an alignment-based distance calculation method. Since its searching capabilities against a database, BLAST is also used as a bioinformatics teaching material. However, when dealing with larger sequences, its performance is poor as it focuses more on aligning regions while discarding the mismatches [7]. These alignment-based methods are highly based on heuristics, and because of that, when some parts are getting aligned other parts can be ignored even if they are similar. Thus these approaches tend to generate less accurate results in certain scenarios. Besides, alignment-based tools need users to do heavy processing steps to make sequences eligible for alignment-based comparisons. Hence it is clear that the use of these implementations makes distance calculations strenuous in practice.

When computing distances between pairs of sequences where one is a repetition of the other, alignment-based methods create inaccurate results. The reason for that is when a particularly common part is aligned, the other pieces get discarded automatically. We can consider Human (Homo sapiens) and Mouse (Mus musculus) genomes as an example in this case. Scientists have figured out that the human genome sequence is a repetition of the mouse genome sequence, and there is high genetic similarity between them. That is why mice are often used as specimens in most of the biological
experiments instead of humans. But when we use alignment-based tools, they tend to provoke lesser similarity, which is not correct compared to actual values. Another reason makes bioinformaticians demotivate on these methods is the high time consumption [8]. Hence, several research studies are conducted to enhance traditional alignment-based distance calculation methods by introducing hardware support[9] and recommendation systems[10].

Alignment-free methods were suggested by bioinformaticians to address the shortcomings in alignment-based methods. These methodologies represent genome sequences as estimations (Time-series distribution / Feature vector, etc.) of the raw genome sequences to speed up the comparison process. Typically, other than distance calculation, alignment-free approaches are used in sequence similarity searches [11], clustering and classification of sequences [12]. Since the lower usage of computational resources, alignment-free methods are named as a better choice for most genome-related comparison experiments. Since these methods are based on subsequence occurrences, they are considered as high memory consuming algorithms [13]. Compared to the alignment-based methods, this kind of approaches is still in the development stage. Therefore, it is clear that to apply alignment-free methods to phylogenetic applications, we need further testing for robustness and scalability.

In present days, alignment-free k-mer and word frequency-based techniques are used in phylogeny applications instead of the genome alignment methods [14]. In a general context, comparison of word frequencies is a smooth process than aligning massive genome sequences which utilizing a high cost of computational resources [15][16]. Feature Frequency Profiles (FFP) of whole genomes is an example of such method in this alignment-free context, which uses word frequencies supported by ‘a variation of a text or book comparison’ method[17]. Composition vector (CV) is another method that computes the normalized frequency of every potential k-mer in given sequences to calculate the genetic relatedness.

In alignment-free approaches, genetic distances are approximated generally with Cosine distance function [18],[19]. Both FFP and CV will interpret the genome sequence using the frequencies of word presence. Moreover, there are some other methodologies, such as spaced-word frequencies to match words for a predefined pattern[20].

Unlike described alignment-free methods, Return Time Distribution (RTU) estimates the genome sequence as a time distribution. Instead of word count, it considers the amount of time required for the reappearance of k-mers [21]. In alignment-free methods, all most all of them are estimating and represent the genome in different formats. Even though these estimation-based genome representations reduce the comparison time, the resultant accuracy is also decreased because of information loss. However, to get a higher accurate genome comparison based on words, we must avoid these estimations and consider both common and distinct k-mers/words of pairwise raw sequences. Yet the problem is this process requires a considerable amount of time and processing as we are comparing raw sequences. Thus when sequences are longer, this method became infeasible.

In our proposed method, we build k-mer forests, which can be used as a direct representation of distinct k-mers in a whole-genome sequence. Distinct k-mers in a species’ genome or a genomic region can be considered as the signature of that species
For the genetic distance calculation, we consider both common and distinct k-mers in each corresponding sequence pairs. Further, we use the Jaccard Index [23] to generate highly accurate distance values that are based on the concept of intersection over the union. As elaborated later in the paper, our method is developed with the expectation of generating the genetic distances with a minimum error when compared to existing alignment-free methods like RTD or FFP. That is because, in the proposed approach, it directly considers similar and dissimilar word counts instead of estimations (e.g., reappearance time-RTD or occurrence patterns-FFP).

Since we do compare genomes using k-mer forests instead of direct scanning through larger sequences for k-mer matching, we have achieved considerably high efficiency. In addition to that, with the help of the pruning algorithm, we developed, we can expect up to 50% speedup in forest comparison. We can guarantee that the proposed methodology provides more accuracy while satisfying the efficiency requirements.

There are several existing methods and tools for phylogenetic tree construction. (IQTree, MEGA). Most of those methods developed by using the neighbour-joining approach. Neighbour-joining is a bottom-to-top approach which means it combines most similar pairs of species and continues to grow the tree upwards. This method inherits some issues. More particularly, in each step, it is required to construct a distance matrix, and this repeated process consumes a considerable amount of time and unnecessary effort leading to reduced efficiency in the process. When getting distances with the combined branches, it takes the arithmetic mean as the new distance which introduces some error. Thus, when distances have less variation, this might lead to the wrong branch joining. Addition to that, if an error occurred, that will propagate to each iteration of the distance matrix. In such scenarios, UPGMA might be failing.

Compared to neighbour-joining methods, our proposed idea is more accurate since it follows a top-to-bottom mechanism by considering all the genetic distances in each of the steps for constructing the tree. Because it considers actual genetic distance instead of arithmetic mean approximation, error propagation does not happen. In addition to that, tree construction is efficient as there is no repeated work as the neighbour-joining method. Because of this, we can mention that our tree construction mechanism is also accurate and efficient than conventional phylogenetic tree construction methods.

3 Research Materials and Datasets

3.1 Genomic datasets

The whole-genome sequences (FASTA format - *.fna) were downloaded from the NCBI database (ftp.ncbi.nlm.nih.gov/genomes/). For the comparisons and results, we used genomes of Bolivian squirrel monkey (ID 6907), Honeybee (ID 48) and North American deer mouse (ID 11397).

For the construction of the phylogenetic tree, we used several genomes of bacteria downloaded from the NCBI database [24].
3.2 Development tools and environmental configurations

For the K-mer counting, we used the DSK k-mer counting tool ([25]). Python was the primary programming language used to implement algorithms. Testing Environment: 32GB RAM, four cores of CPU, 1TB Storage, Ubuntu 16.04

After downloading the corresponding genome datasets from NCBI, we extracted k-mers using the DSK tool. The output contains a list of k-mer strings and their frequencies. We converted that output to CSV (Comma Separated Values) to achieve the ubiquitoussness and compactness of data.

4 Methodology

To address the issues mentioned above, we proposed a novel method of genetic distance calculation and phylogenetic tree construction.

The proposed method of genetic distance calculation is based on k-mer count (word count), and it is an alignment-free technique. K-mers are substrings that have a length of k. As we have discussed in the background section, existing alignment-free methods such as Feature frequency profiles (FFP) [17], Composition vector (CV) and Return time distribution (RTD) use different of estimations to represent the genomes, which reduce the accuracy in distance calculation. Additionally, these methods are failing to consider common and distinct k-mers in relevant sequences that are required to give high precision in comparison. However, with our approach of generating the k-mer forests, we can represent large whole-genome sequences in a compact and simplified manner, which helps us to consider common and distinct substrings conveniently.

More particularly, with this method, we can expect high accuracy as well as efficiency. Calculating genetic distance consists of two major steps, explained in Part A and Part B below. At the end of those two steps, the distance matrix can be generated. Then, we move to Part C, i.e. phylogenetic tree construction using those distances.

4.1 Part A - Creating k-mer forests for sequences

The initial step of genetic distance calculation is constructing k-mer forests for each of the whole genome sequences. To build the forest, initially, it is required to list all distinct k-mers of the genome sequence. To do that, we used DSK (disk streaming of k-mers) k-mer counting software which lists k-mers with a considerable low memory and disk usage [25]. After collecting the distinct k-mers, the k-mer forest is built, as explained in the flowchart in Fig. 2.

The output of the algorithm explained in Fig. 2 is the k-mer forest, which is built using all the distinct k-mers listed using the DSK tool. Each root-to-leaf path in the forest represents a distinct k-mer in the sequence. Because of this, each tree in the k-mer forest is only k-deep, which is very concise and informative. Not only that, when we consider nucleotide sequences the maximum possible number of trees is 4, as alphabet size is 4 with A, C, T, G as their roots. If protein sequences are used, then the max-
imurn number of trees became 20 as alphabet size is 20. Hence ultimately massive genome sequences are converted to a simplified structure, which is straightforward to compare the genetic distance with this k-mer forest construction method. Figure 1 shows an example of constructing a k-mer forest for a given sequence.

**DNA Sequence** - ACGTGACCCTTA

All k-mers where k=4 – ACGT, CGTG, GTGA, TGAC, GACC, ACCC, CCTT, CTTA

k-mer forest -

![Forest Diagram]

**Fig. 1.** Building a k-mer forest for an example sequence

In the above example (Fig. 1) of forest construction, there are nine distinct k-mers with k=4 in the corresponding DNA sequence. For that set of distinct k-mers, the forest is built with four trees. Even though the sequence is large if we use this configuration,
still we get this size of forest (with max four trees), and each tree's depth is also k. Because of this approach, even though the genome sequence is several billion characters long, still we get a forest of max size equal to alphabet size and max tree depth equal to value k. It is expected to perform better than comparing naked genome sequences to find common and distinct k-mers.

4.2 Part B - Calculate distances using the tree comparison algorithm

After completing Part A, we have successfully converted the large DNA sequences to concise k-mer forests. In this second stage, we compare those forests to get the genetic distance between species. To do that, we are proposing a novel tree comparison algorithm supported with tree pruning. With that, forests are compared with optimal traversal and minimum time consumption. Following algorithm 1 shows the implementation of tree comparison mechanism.

| Algorithm 1: Get distance between two forests |
| Require: Forest 1 (F1), Forest 2 (F2) |
| 1. FUNCTION get_distance (F1, F2) |
| 2. LET D = Number of pathways F1 has F2 doesn’t |
| 3. LET S = Number of common pathways in forests |
| 4. FOR each node in F1 |
| 5. IF node not in F2 nodes |
| 6. IF node is empty |
| 7. D += 1 |
| 8. ELSE |
| 9. D = get_child_count(node) |
| 10. ELSE |
| 11. IF node is empty |
| 12. S += 1 |
| 13. get_distance (F1[node], F2[node]) |
| 14. END FOR |
| 15. LET U = Number of all pathways in forests |
| 16. LET J_1 = Jaccard index |
| 17. U = D = F2.kmer_count |
| 18. J_1 = k/U |
| 19. Return J_1 |
| 20. END FUNCTION |

Here in pruning based comparison, instead of traversing entire forests from root to leaves to find common and distinct k-mers, we can prune certain tree sections based on identification in higher levels. With that, the amount of work reduced by a considerable amount. When we compare two forests, it happens level by level from root to leaves. If forest A is compared with forest B, this algorithm returns Jaccard similarity between A and B. Pruning happens once any node is encountered uncommon and then, without traversing through children of that node in the second tree, using a recursive function [26] add the child count to the distance directly (uncommon k-mer count). With that, efficiency is drastically improved as no need for traversing children of such a node.

As an instance, tree pruning is explained in Figure 3. When our comparison algorithm is comparing forest I and forest II, the algorithm is detecting that Node A (with
parent C) is present in the forest I but not in forest II. Thus, here pruning taking place and child count of that Node A is directly added to the distance without traversing through its children nodes (circled in Fig. 3).

![Forest I and Forest II](image)

**Fig. 3.** k-mer forest pruning

Ultimately here it identifies there are no any kmer starting with “CA” in Forest II and rapidly get distance updated from “CA” starting k-mers in Forest I.

\[
    \text{Distance} = \frac{\text{Number of common pathways in forests}}{\text{Number of all pathways in forests}}
\]

![Venn diagram](image)

**Fig. 4.** Venn diagram showing the pathways of forest A and B

Here the term pathway stands for a root to leaf routine in a tree in the forest. In other words, it is a k-mer in the genome sequence. As explained above, if comparing two forests A and B, as an initial step, we calculate the number of pathways only presented in A (indicated as D in Fig. 4). The common pathways can be calculated by merely subtracting this value from the total k-mer count in A: shown as S in the diagram. This gives the numerator for the Jaccard equation. Then we can find the number of all pathways in forests by adding D to the k-mer count in B, (i.e., the denominator of the Jaccard equation) and then we can compute the Jaccard relatedness of the two species.
In summary, once we get set of genome sequences to construct the phylogenetic tree, we build k-mer forest for each of those sequences using the method in Part A. Afterwards, we compare each of those forests and calculate all-to-all distances using the technique explained in Part B. The outcome of these two stages is an all-to-all distance matrix, which consists of genetic relatedness among the given species set.

4.3 Part C - Creating phylogenetic tree

After completing Part A and Part B, the distance matrix is made, and the next step is to construct the phylogenetic tree based on these distances. To do that, we used an algorithm built on top of k-medoid clustering. That can overcome the problems we have in conventional neighbour-joining tree construction approaches. Algorithm 2 shows the method we used to construct the tree.

Here we use the k medoid algorithm by taking K as 2. First, we are dividing the entire species set to two clusters by considering all to all distances from the distance matrix. Afterwards, we iteratively apply the same process to those two clusters and separate each into another two clusters as all together we get 4 clusters. This process is repeated until a single species belongs to one cluster. In the algorithm, we have satisfied this by recursive techniques.

This method can be further explained by the following diagram (Fig. 5). Here species are plotted by considering all to all distances in the distance matrix. Each diamond with different colour represents a species. First, the species set is clustered into two as showed in two large circles. Then each of the species within those clusters is further divided. The resulted tree is made as following (Figure 6).
5 Results and Evaluations

In this section, we present the results and evaluations we made on our genetic distance calculation and phylogenetic tree construction methods. When considering the k-mer forest construction, one of the crucial decisions is to select the best appropriate k value to extract k-mers. When we are using different k values, the number of distinct k-mers can be obtained from the sequence is changing. When the number of distinct k-mers is increasing, it can describe the genome sequence better. Thus, we experimented to figure out the best suitable value to construct the k-mer forest to get better results.

For that, we select different k values and compare the number of distinct k-mer counts extracted. The following graph (Fig. 7) shows those results. When we increase the k value, the distinct k-mer count reaches a peak and then gradually reduces. The k value that gives the peak can be considered as the best k value to extract the k-mers from the sequence. In this case, the optimal k value is 15, as shown in the graph. For
this particular experiment, we have chosen the whole DNA sequence of Apis mellifera (Honeybee).

![Fig. 7. Distinct k-mer count vs k-mer size graph](image)

In addition to that, we have evaluated how forest comparison time varies with the different k values of the genome. For this experiment too, we have used the k-mer forest of species Apis mellifera. The comparison is made against itself to get the worst-case comparison (no pruning).

When comparing this graph with the previous graph, it shows a similar nature and meets the peak point at 15. Hence, this explains that forest comparison time-varying proportionally to the number of distinct k-mers.

Fig 8 shows the worst-case forest comparison time at the peak; still, it takes only about 50 seconds to make the entire forest comparison. Hence, this is again considerably high performance compared to other alignment-free approaches.

![Fig. 8. Forest comparison time vs k-size graph](image)
5.1 Performance of tree comparison algorithm – Pruning

As we discussed above, the tree comparison algorithm is based on tree pruning. If a particular node does not exist in the other forest without traversing through its child nodes, all subroutines of that node will be added to the distance using Algorithm 3. With this, tree comparison time can be improved by up to 50%.

The following table shows the percentage of speed-up in comparison when pruning occurs. For this, we have taken the 13-mer forest of Peromyscus maniculatus which consists of 67108864 distinct k-mers in 4 trees. There are five cases, and each of those cases forest is compared with itself by removing trees. Speed up is calculated concerning the non-pruning case [26].

| Tree count in A | Trees count in B | Pruned tree count | Time taken (s) | % speedup |
|----------------|-----------------|-------------------|----------------|-----------|
| 4              | 4               | 0 (non-pruning case) | 28.927         | 0%        |
| 4              | 3               | 1 (25%)           | 27.413         | 5.25%     |
| 4              | 2               | 2 (50%)           | 24.743         | 14.48%    |
| 4              | 1               | 3 (75%)           | 20.326         | 14.93%    |
| 4              | 0               | 4(all trees are pruned) | 14.408         | 50.19%    |

Table 1. Comparison time speedup with pruning

5.2 Phylogenetic tree construction

Using the distance matrix we built at the end of steps A and B, we made the phylogenetic tree using our modified version of the k-medoid algorithm. Here we have used 10 Bacteria as our species set and the phylogenetic tree is constructed as follows (Figure 9). Bacteria set is used as genetic distance variance is extremely low, and high sensitivity is required to cluster them. Our method has successfully built the forest as below.

![Phylogenetic tree for a set of ten bacteria](image-url)
When comparing the above tree with the NCBI taxonomy, this shows better accuracy compared to the neighbour-joining method as bacteria belong to the same kingdom, are in the same sub-branches. Reason for that is when getting arithmetic means in less variant distances results mismatches. Therefore, when comparing the tree constructed by neighbour-joining with the taxonomy, we can justify that our method is performed at an increased accuracy.

5.3 Accuracy of genetic distances and phylogenetic tree

For assessing the accuracy of the genetic distances, either we must evaluate our results with globally recognized ground truths or use distance values calculated by other existing methods. When considering the existing genetic distance calculation methods, they use their techniques to generate distances.

Because of the specificity of the existing methods, we cannot compare the distances since they belong to different scales and interpretations. However, one good way to evaluate the accuracy of distances is to compare relative distances between species from different methods. To do that, the best approach is to compare phylogenetic trees generated from different methods as they are a direct indication of relative distances. At the same time, we cannot guarantee that the existing methods such as FFP, RTU, BLAST can provide us high accurate phylogenetic trees because of their inherited issues as we explained in the background section of the paper.

We believed that to evaluate the accuracy of our method, a globally recognized ground truth as the benchmark must be used. Thus, we validated our approach by taking the species’ taxonomy as the ground truth. The Taxonomy was obtained using NCBI’s Taxonomy Browser [27]. The following figure shows how our constructed phylogenetic tree matches with the taxonomy as different branches show different sub-kingdoms in the taxonomy. However, it shows that these results may not be 100% accurate with respect to the complete taxonomy. Mismatching appeared during the comparison are emphasized in Red (Fig. 8). Some species in the same kingdom have miscategorized and put into different branches. In our method, we consider genetic distances. Yet there can be species in the same kingdom who are genetically diverse (e.g., Rat vs Human). Thus this can be a reason for such miscategorising.
When considering tree construction performance, as not required to construct the distance matrix in each step, our method has comparatively small phylogenetic tree construction time. As explained by the following diagram (Fig. 9), forest construction time increased with the number of species. Here when constructing a phylogenetic tree using all 10 species only required about 600 ms.

### Discussion and Conclusion

From this paper, we have proposed a novel method of genetic distance calculation and phylogenetic tree construction. In the genetic distance calculation method, one of the most critical decisions was to select the optimal value for $k$, which gives the best accuracy in distance calculation. The reason for that is, at the optimal value of $k$ maximum number of k-mers can be extracted. With that, we can construct the highest sensitive k-mer forest.
When comparing our genetic distance calculation method to other existing methods, our method outperforms both the accuracy and efficiency-wise. The reason behind that is in our approach we consider the direct representations to genome sequences by k-mer forests instead of estimations. With the help of tree comparison and pruning mechanism, the efficiency is further increased.

The outcome of the genetic distance calculation method is to construct the distance matrix. The phylogenetic tree is made based on this distance matrix. Our way of phylogenetic tree construction is efficient and accurate in contrast to most of the existing neighbour-joining based tree construction methods. Avoidance the repeated work, error propagation, and using a top-down approach is the reason behind it.

As the next stage of our research, we are planning to enhance the accuracy of the genetic distance calculation method by considering the kmer frequency. We planned to optimized tree pruning by keeping child count at each internal node. So in situations like case 5 in pruning performance table, we can complete comparison in near-zero time. Further, we are working on proposing a neural network to add new species to the already constructed phylogenetic trees efficiently. Addition to that it is expected to increase the performance of genetic distance calculation tree construction methods by integrating GPU capabilities [28]. Once the entire phylogenetic tree construction and updating workflow is built, it is expected to integrated with our existing bioinformatics learning workflow[29].

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