

## Article

# Phylogenetic Tree Selection by Testing Substitution Number in Clade

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**Abstract:** The phylogenetic tree is a widely-used tool to show the evolutionary relationship between taxa. There are many types of phylogenetic trees proposed in the literature such as maximum likelihood, neighbor-joining, and UPGMA trees. The topologies of different types of trees are not the same. Even for the same type of tree, the topologies are different when they embed different nucleotide substitution models, such as the JC69 model, K80 model, TN93 model, and so on. Although each type of tree has its advantages, selecting a suitable tree among these choices becomes a challenging problem. In this study, we propose a method based on testing the nucleotide substitution number between sequences to select trees. An Ebolavirus example is used to illustrate the method. In addition, this approach can select a suitable nucleotide substitution model for a particular type of tree. For this ebolavirus example, the JC69 model is the selected substitution model for the maximum likelihood tree.

**Keywords:** phylogenetic tree; nucleotide substitution model; *p*-value; substitution number



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## 1. Introduction

The phylogenetic tree is a useful method to show the relationship between different taxa [1]. The branching relationships of a tree can reflect the degree to which different sequences are related. Two sequences that are very much alike will be located as neighboring outside branches. In addition to displaying the relationship between different taxa, the phylogenetic tree is a useful method to predict the microRNA (miRNA) biomarkers of cancers by combining other bioinformatics approaches [2]. Furthermore, the phylogenetic tree has been used as a very useful tool to explore the association between various diseases and the association between disease and vaccination. The association between major depression and gastroesophageal reflux has been explored based on the phylogenetic analysis of miRNA biomarkers [3]. Additionally, the association between anti-NMDA receptor encephalitis and vaccination [4,5] and the association between anti-NMDA receptor encephalitis and the coronavirus disease 2019 (COVID-19) have been explored using the phylogenetic analysis [6].

Many types of trees have been established in the literature, including the maximum likelihood method, the neighbor-joining method, the unweighted pair-group method using arithmetic averages (UPGMA), and the maximum parsimony method [7–10]. They are either based on a character criterion or a distance criterion. For example, the maximum parsimony tree and the maximum likelihood tree are character-based methods. The UPGMA and neighbor-joining trees are distance-based methods. There are two types of error for phylogenetic trees including the topological error and the branch-length error [11]. The topological error is the difference in the branching pattern between an inferred tree and the true tree, and the branch-length error is the deviation of estimated branch lengths from the true branch lengths. Topological errors might be more serious than branch-length errors. Even for the same type of tree, the topology is not exactly the same when embedding different substitution models. Although each type of tree has its advantages, different trees

may have very distinct topologies. Thus, we might be interested in selecting trees satisfying some topology criterion.

Several approaches have been developed to assess the confidence of tree selection including the bootstrap probability [12], the Kishino–Hasegawa tests [13,14], and an approximately unbiased test [15]. The approximately unbiased test was recommended for the tree selection problem, which could satisfy the requirement for unbiasedness at least approximately and thus controls for type-1 error in most cases [15]. In addition, a k-means clustering method was also proposed to select a suitable tree [16]. The k-means clustering method does not need any evolutionary model assumptions, but only needs to investigate sequence similarity using a penalty score. However, the calculation process of this approach is complicated, which might not be very useful in real applications.

In this study, we propose using the method of testing substitution number per site to select phylogenetic trees. This method is based on the topology of the tree. The substitution number between the nucleotide sequences in the same clade of a reasonable tree should be smaller than the substitution number between the nucleotide sequences in different clades. Thus, we use this criterion to propose our method, and regard the selected trees as suitable trees. It is noted that this method is proposed from an intuitive point of view that the nucleotide sequences in the same clade should be more similar to those in different clades. Nevertheless, there is no guarantee that this method will always select the true tree. The criterion of this method requires that the substitution number per site be small between two sequences joined to a common branch. Since the estimated substitution number per site is a point estimator that cannot provide any estimation precision, to provide a more precise method to test it, we adopt a hypothesis testing to test whether the substitution number per site for the sequences joined to a common branch is zero.

To propose the hypothesis testing, we need to choose a nucleotide substitution model for sequence evolution. There are many kinds of nucleotide substitution models, including the JC69 model, K80 model, F81 model, F84 model, HKY85 model and TN93 model [7,17–21]. The substitution number estimator and its variance estimator for these substitution models have been well established in the literature [17,18,22–26]. From our studies, in developing the method, the nucleotide substitution model selection does not significantly affect the tree selection result. Therefore, we adopt the simple JC69 model as the substitution model in the hypothesis testing. An Ebolavirus example is used to illustrate and validate the method. To plot a phylogenetic tree for the Ebolavirus by the software MEGA 6 [27], first, we need to select a tree construction method such as the maximum likelihood, neighbor-joining, or UPGMA method. After that, we need to select a substitution model to plot the tree.

## 2. Methods

Before describing the method, we introduce notations first. Let  $d$ ,  $\hat{d}$  and  $\hat{V}(d)$  denote the substitution number, substitution number estimate, and variance estimate, respectively. To compare different trees, we adopt the similarity of sequences in the same clade of trees to evaluate the performance of the trees. To measure the similarity of two sequences, we can use the  $p$ -value of the hypothesis test,

$$H_0 : d = 0 \text{ versus } H_1 : d > 0, \quad (1)$$

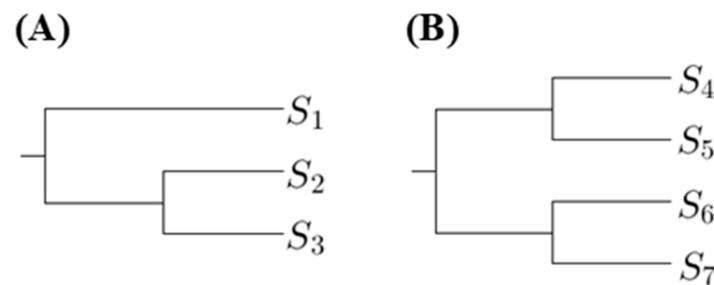
to be a similarity degree of two sequences. A large  $p$ -value for testing (1) indicates more similarity between the two sequences because the null hypothesis  $H_0$  is not rejected. The  $p$ -value for testing (1) is:

$$p\text{-value} = \Pr\left(Z > \frac{\hat{d}}{\hat{V}(d)}\right), \quad (2)$$

where  $Z$  is the standard normal random variable. When two sequences are the same, since  $\hat{V}(d)$  does not exist, we define the  $p$ -value to be 1. Here the normal approximation is considered in (2). Since the length of the gene nucleotide sequence of a gene is usually large,

by the central limit theorem, we can assume that the substitution number follows a normal distribution. If the length of the nucleotide sequence is not large such as the microRNA nucleotide sequence, we might consider other tests.

The proposed method is to select a tree such that the p-values for any pairs of sequences in the same clade are not too small. Hence we first compute the number of p-values less than a threshold in the first clades. Using Figure 1 as an example, there are two phylogenetic trees, where  $S_1, \dots, S_7$  are DNA sequences. Let  $d_{S_i, S_j}$  denote the substitution number of the two sequences  $S_i$  and  $S_j$  for  $i \neq j$ . For Figure 1A, we compute the p-value for testing  $H_0 : d_{S_2, S_3} = 0$  in the first clade; for Figure 1B, we compute the p-values for testing  $H_0 : d_{S_4, S_5} = 0$  and  $H_0 : d_{S_6, S_7} = 0$  in the first clade.



**Figure 1.** Two phylogenetic trees showing first clades (A) and second clades (B).

The phylogenetic trees, which have the minimum number of p-values less than a threshold in the first clades, are the most suitable. If there is more than one tree achieving this criterion and we want to select one, we can compare the number of p-values in second clades. There are two types of the second clade presented in Figure 1. When comparing the p-values in second clades, we compute the p-values for testing two sequences that are from distinct first clades. Using Figure 1 as an example, for Figure 1A, we compute the p-values for testing  $H_0 : d_{S_1, S_2} = 0$  and  $H_0 : d_{S_1, S_3} = 0$ ; for Figure 1B, we compute the p-values for testing  $H_0 : d_{S_4, S_6} = 0$ ,  $H_0 : d_{S_4, S_7} = 0$ ,  $H_0 : d_{S_5, S_6} = 0$  and  $H_0 : d_{S_5, S_7} = 0$ . Then we choose the tree with the minimum number of p-values less than a threshold in second clades, and so on.

To simplify the notation, we define the number of p-values less than a threshold  $k$  in the  $i$ th clades as  $N(i, k)$ . However, only comparing the number of p-values less than a threshold is not an appropriate way to select a tree. Instead, a more reasonable approach is to compare the ratio of p-values less than a threshold. For example, when considering the second clade cases in Figure 1, the number of all p-values for Figure 1A is 2 and the number of all p-values for Figure 1B is 4. Hence we cannot directly compare the number of p-values less than the threshold because the number of p-values for each tree is not the same. Therefore, for the  $i$ th clade, we define a magnitude,

$$R(i, k) = \frac{\text{the number of } p\text{-values in the } i\text{th clade which are less than a threshold } k}{\text{the number of } p\text{-values in the } i\text{th clade}}, \quad (3)$$

which is the ratio of the number of p-values in this clade less than the threshold to the number of p-values in this clade. It is noted that we do not need to consider multiple testing to test several hypotheses here because Equation (3) is used instead of using the number of p-values less than the threshold. This is somehow equivalent to adjusting the p-values. A procedure for selecting a tree based on  $R(i, k)$  is given as follows.

Procedure 1: Select a suitable phylogenetic tree

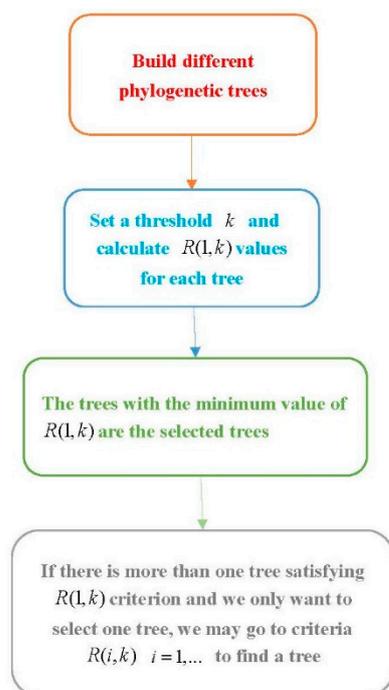
Step 1. Use software to build several phylogenetic trees using different methods.

Step 2. Set a threshold  $k$  and compute  $R(1, k)$  for each phylogenetic tree.

Step 3. The trees with the minimum value  $R(1, k)$  are the desirable trees under the  $R(1, k)$  criterion. If there is more than one tree with the minimum  $R(1, k)$  value, we can go to the next steps.

Step 4. Let  $i = 2$ . For the trees with the minimum value of  $R(i - 1, k)$ , compute  $R(i, k)$  and follow a similar way in Step 3 to select a tree. If there is more than one tree satisfying the condition, we go further to consider the  $(i + 1)$ th clade and follow a similar method to select trees.

This method can be easily implemented when we only consider the  $R(1, k)$  criterion. The criteria of  $R(i, k)$  with  $i \geq 2$  are more difficult to carry out. Although only using the  $R(1, k)$  criterion might select more than one tree, it is easier to carry out. The flowchart of this procedure is provided in Figure 2.



**Figure 2.** The flowchart of Procedure 1.

In Procedure 1, the threshold  $k$  should be determined first. Since the threshold of the p-value should be small, we suggest that  $k$  can be in the range of  $(0, 0.1)$ . A more appropriate way is to consider several values of  $k$  and select a tree by comparing the results for these different  $k$  values. More details of  $k$  selection are discussed in Section 3. In addition, it is worth noting that there are many options for the variance estimator  $\hat{V}(d)$  in Equation (2) [24,26]. According to our study with the Ebolavirus example, different nucleotide substitution models and different variance estimators do not affect the result. Thus, in the example, we adopt the substitution model JC69 and its usual variance estimator.

$$\hat{V}(d) = \frac{\hat{d} - \hat{d}^2}{n(1 - (4/3)\hat{d})^2}, \quad (4)$$

where  $n$  is the length of the sequences.

### 3. An illustrate Example and Result

We use an Ebolavirus example to illustrate our method. The genus Ebolavirus contains five species: Bundibugyo ebolavirus (BDBV), Zaire ebolavirus (EBOV), Reston ebolavirus (RESTV), Sudan ebolavirus (SUDV), and Tai Forest ebolavirus (TAFV) [28]. The three species—BDBV, EBOV and SUDV—have been associated with large Ebolavirus disease outbreaks in Africa. EBOV and SUDV are the main viruses of the five Ebolaviruses. Hence in this study, we discuss 28 Ebolaviruses in these two species. The accession numbers of the Ebolaviruses in the Genbank are AF272001.1, AY354458.1, EU338380.1, FJ968794.1,

HQ613402.1, HQ613403.1, JN638998.1, KC242783.2, KC242784.1 to KC242794.1, KC242796.1, KC242798.1 to KC242801.1, KJ660346.2 to KJ660348.2, and NC\_006432. The Ebolaviruses EU338380, FJ968794, JN638998, KC242783, and NC\_006432.1 belong to the SUDV species and the other Ebolaviruses belong to the EBOV species.

Figures 3–5 show the maximum likelihood trees, neighbor-joining trees, and UPGMA trees plotted by MEGA 6 with distinct substitution models for these 28 Ebolaviruses. The maximum likelihood trees in Figure 3A–C are based on the JC69 model, K80 model, and Tamura 3-parameter model, respectively. The neighbor-joining trees in Figure 4A–D are based on the number of the differences between two sequences, the p-distance, JC69 model, and the maximum composite likelihood model, respectively. The UPGMA trees in Figure 5A,B are based on the JC69 model and TN93 model.

Here we follow Procedure 1 to choose the suitable tree and use the maximum likelihood tree based on the JC69 model as an example in Figure 6. We compute the p-values in all of the first clade. For example, Figure 6 shows the p-values of the first clade for the maximum likelihood tree based on the JC69 model.

After computing the p-values for all of the first clades, we can calculate the  $R(1, k)$  values that are provided in Table 1.

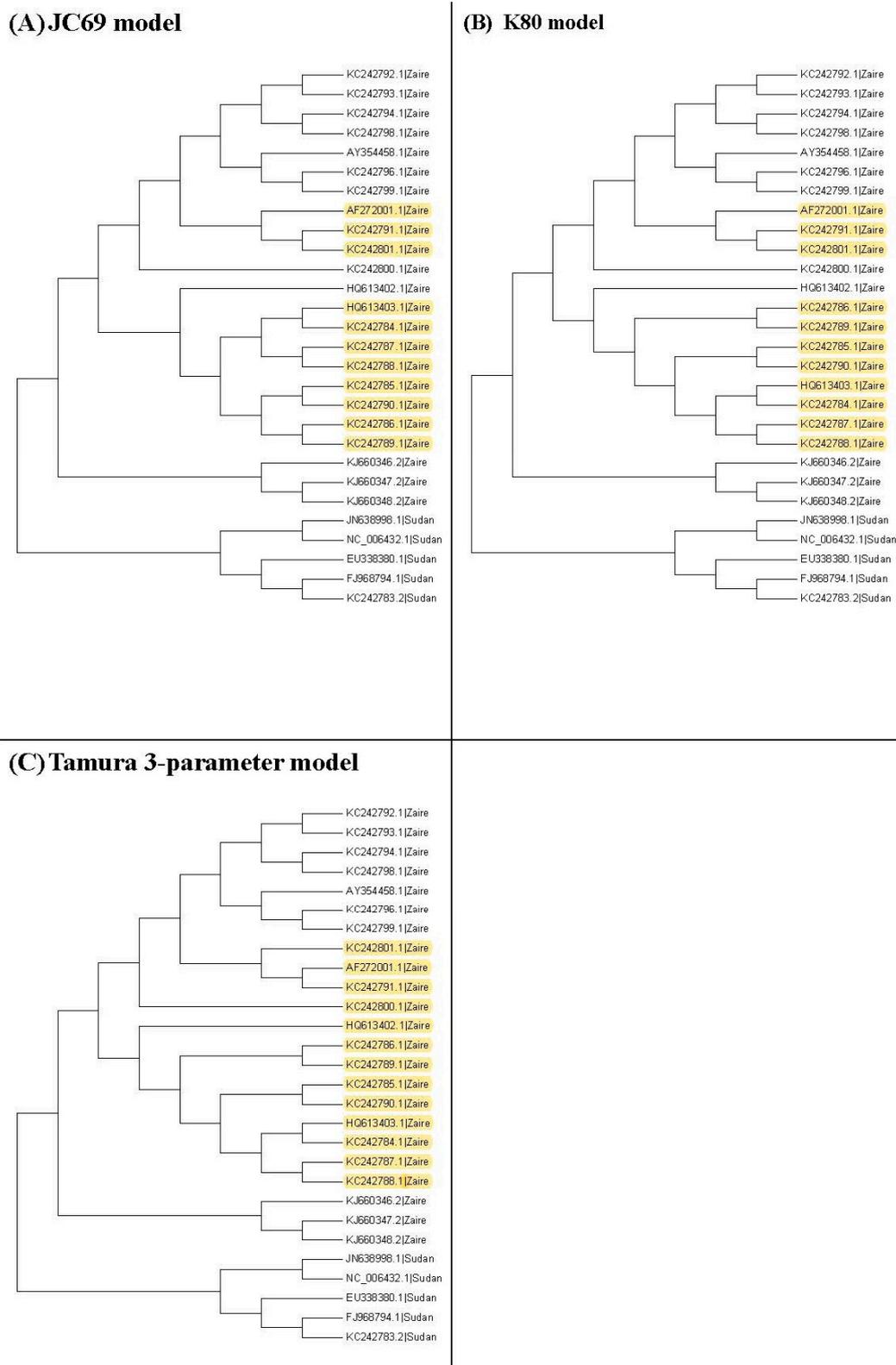
**Table 1.** The  $R(1, k)$  values for phylogenetic trees.

		<i>k</i>									
Substitution Model		0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
Maximum likelihood	JC69 model	$\frac{5}{11}$	$\frac{5}{11}$	$\frac{6}{11}$	$\frac{6}{11}$	$\frac{8}{11}$	$\frac{8}{11}$	$\frac{8}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$
	K80 model	$\frac{5}{11}$	$\frac{5}{11}$	$\frac{6}{11}$	$\frac{6}{11}$	$\frac{8}{11}$	$\frac{8}{11}$	$\frac{8}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$
	Tamura 3-parameter model	$\frac{6}{11}$	$\frac{6}{11}$	$\frac{7}{11}$	$\frac{7}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$
Neighbor-joining	No. of differences	$\frac{5}{11}$	$\frac{5}{11}$	$\frac{6}{11}$	$\frac{6}{11}$	$\frac{8}{11}$	$\frac{8}{11}$	$\frac{8}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$
	p-distance	$\frac{5}{11}$	$\frac{6}{11}$	$\frac{6}{11}$	$\frac{6}{11}$	$\frac{8}{11}$	$\frac{8}{11}$	$\frac{8}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$
	JC69 model	$\frac{6}{11}$	$\frac{7}{11}$	$\frac{7}{11}$	$\frac{7}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$
	Maximum composite likelihood	$\frac{6}{11}$	$\frac{7}{11}$	$\frac{7}{11}$	$\frac{7}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$	$\frac{9}{11}$
UPGMA	JC69 model	$\frac{3}{9}$	$\frac{3}{9}$	$\frac{3}{9}$	$\frac{3}{9}$	$\frac{5}{9}$	$\frac{5}{9}$	$\frac{5}{9}$	$\frac{7}{9}$	$\frac{7}{9}$	$\frac{7}{9}$
	TN93 model	$\frac{3}{8}$	$\frac{3}{8}$	$\frac{3}{8}$	$\frac{3}{8}$	$\frac{4}{8}$	$\frac{4}{8}$	$\frac{4}{8}$	$\frac{6}{8}$	$\frac{6}{8}$	$\frac{6}{8}$

In this study, we consider several  $k$  values. For these  $k$  values, we find that the trees based on the JC69 model and K80 model have the minimum value of  $R(1, k)$ . Therefore, according to the  $R(1, k)$  criterion, these two trees are desirable. We can further compare these two trees by using the  $R(2, k)$  criterion. Hence we further consider the second clades for these two trees. Figure 7 shows all the second clades for them. Then we find that the second clades are the same for these two trees except for one (marked with yellow color), which appears in the tree based on the JC69 model, but not in the tree based on the K80 model. In this case, according to the criterion, this method cannot be applied to compare the p-values in the second clade. The reason is that we need to compare the difference between these two trees in Figure 7. Only the subtree marked with the yellow color is the difference between these two trees. We can calculate  $R(2, k)$  for this subtree marked with the yellow color, but there is no corresponding subtree in the K80 model that we can calculate its  $R(2, k)$  value. Therefore, we cannot compare them for the second clade.

Thus, we go further to the third clades to compare these two trees. Figure 8 shows all the third clades for them and only one clade is different. Tables 2 and 3 show the p-values for testing the nucleotide substitution numbers between sequences in the third clade for the maximum likelihood trees based on the JC69 model and the K80 model, respectively. Table 4 shows  $R(3, k)$  values for several thresholds  $k$ . We find that the tree based on the

JC69 model has the minimum value  $R(3, k)$  for these  $k$ 's. Then, according to our method, this tree is the selected one.



**Figure 3.** The maximum likelihood trees for the 28 Ebolavirus sequences based on the JC69 model, K80 model, and Tamura 3-parameter model. Yellow color: difference.

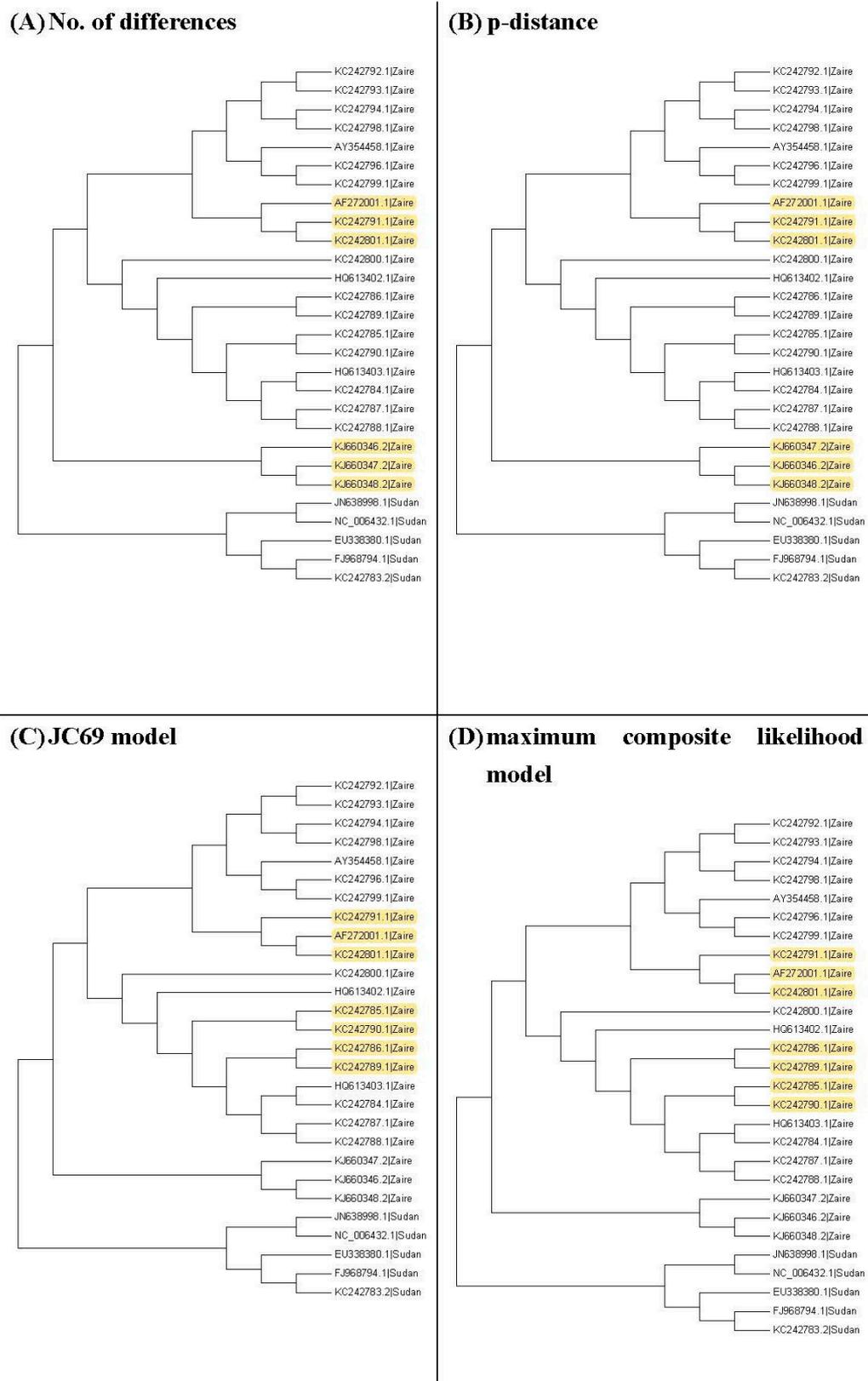


Figure 4. The neighbor-joining trees for the 28 Ebolavirus sequences. Yellow color: difference.

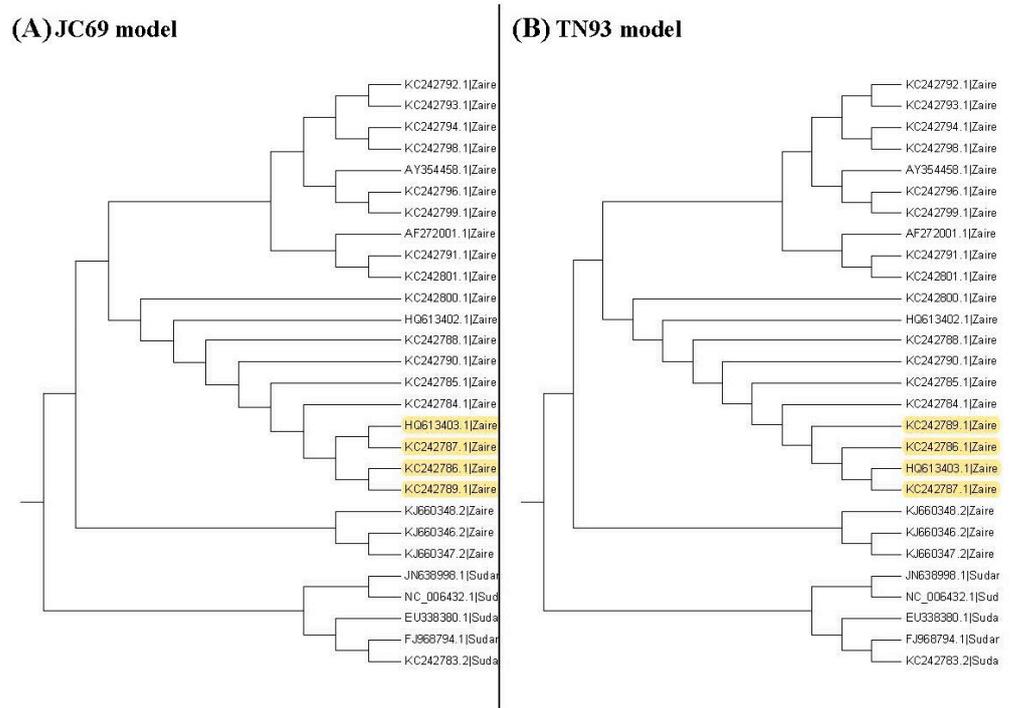


Figure 5. The UPGMA trees for the Ebolavirus sequences. Yellow color: difference.

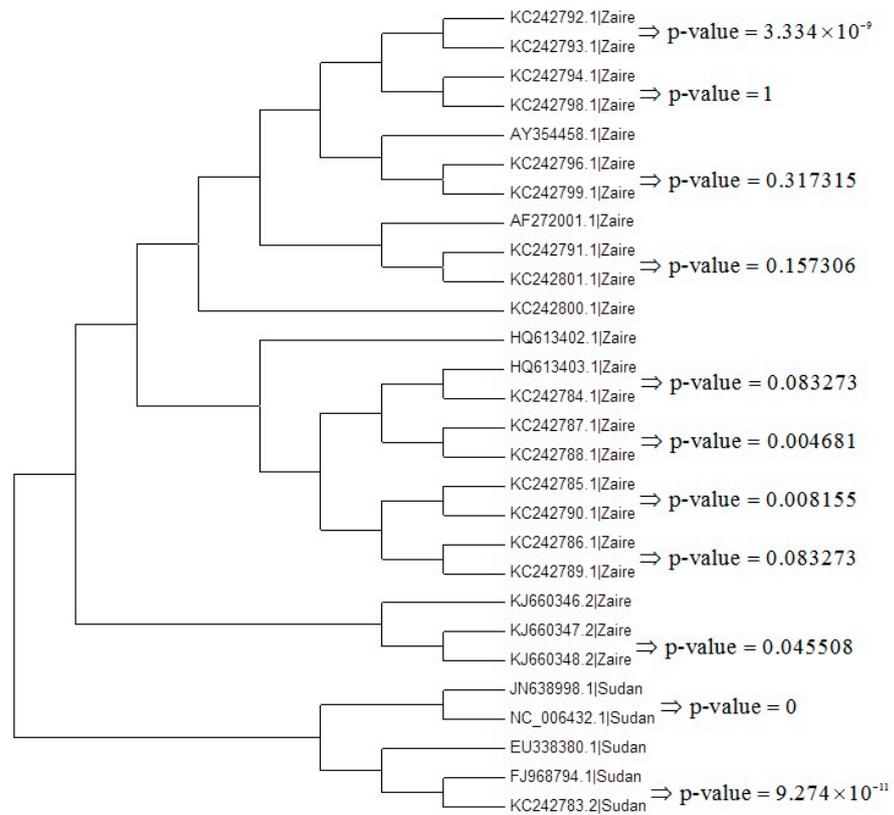
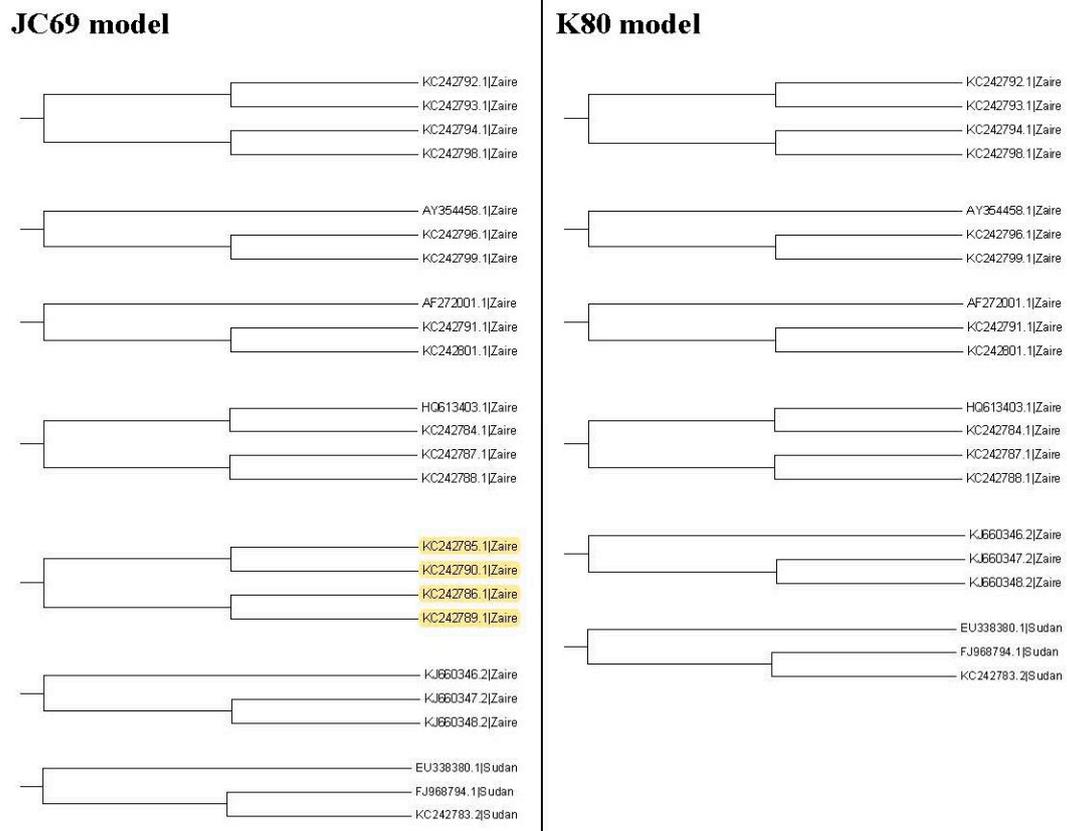
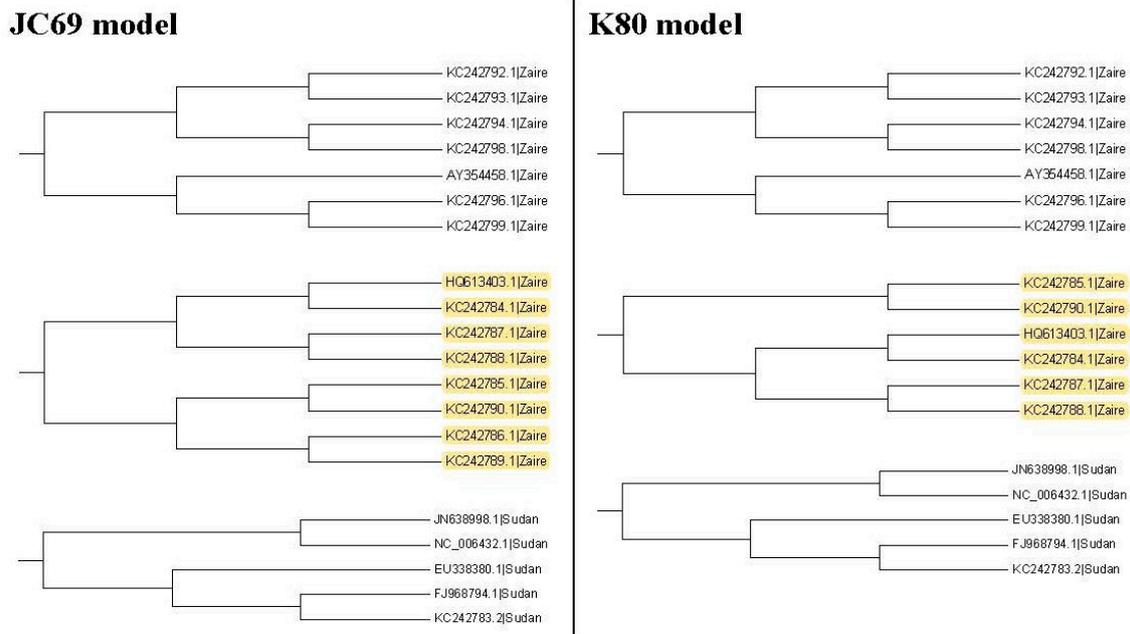


Figure 6. The p-values for testing the nucleotide substitution number between sequences in the first clades for the maximum likelihood tree based on the JC69 model.



**Figure 7.** The second clades for the maximum likelihood trees based on the JC69 model and the K80 model. Yellow color: difference.



**Figure 8.** The third clades for the maximum likelihood trees based on the JC69 model and the K80 model. Yellow color: difference.

**Table 2.** The p-values for testing the nucleotide substitution number between sequences in the third clade for the maximum likelihood tree based on the JC69 model.

	HQ613403.1   Zaire	KC242784.1   Zaire	KC242787.1   Zaire	KC242788.1   Zaire
KC242785.1   Zaire	0.012677	0.002341	0.012677	0.000156
KC242790.1   Zaire	0.007156	0.001351	0.007156	0.000092
KC242786.1   Zaire	0.041637	0.007156	0.041637	0.000456
KC242789.1   Zaire	0.022754	0.004078	0.022754	0.000267

**Table 3.** The p-values for testing the nucleotide substitution number between sequences in the third clade for the maximum likelihood tree based on the K80 model.

	HQ613403.1   Zaire	KC242784.1   Zaire	KC242787.1   Zaire	KC242788.1   Zaire
KC242785.1   Zaire	0.012677	0.002341	0.012677	0.000156
KC242790.1   Zaire	0.007156	0.001352	0.007156	0.000092

**Table 4.** The values  $R(3, k)$  for the maximum likelihood tree. The difference between these two models is marked by the red color in the JC69 model.

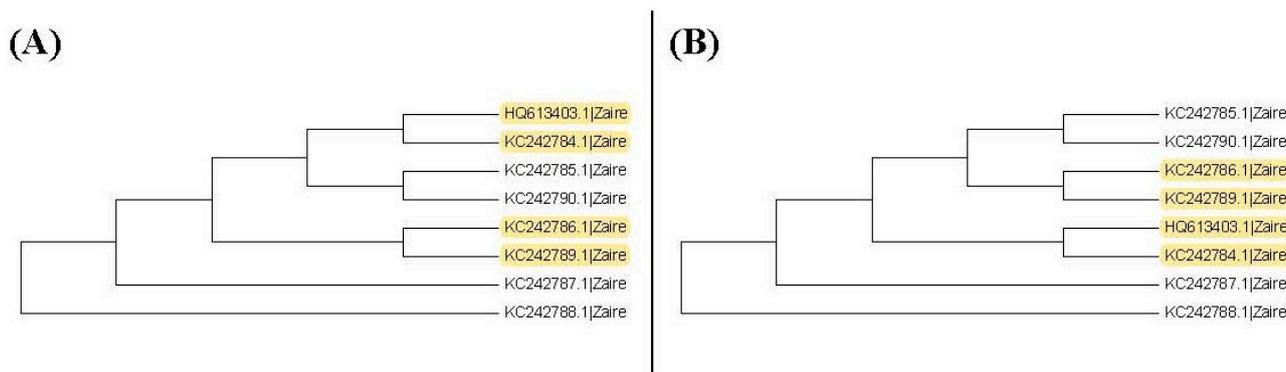
Substitution Model	$k$									
	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
JC69 model	$\frac{10}{16}$	$\frac{12}{16}$	$\frac{14}{16}$	$\frac{14}{16}$	$\frac{16}{16}$	$\frac{16}{16}$	$\frac{16}{16}$	$\frac{16}{16}$	$\frac{16}{16}$	$\frac{16}{16}$
K80 model	$\frac{6}{8}$	$\frac{8}{8}$								

In this example, we can see that the proposed method cannot be applied in the second clade. It is easier to calculate all of the p-values for the first clade. For the second or upper clades, it is not as easy as the case in the first clade to calculate all of the p-values. Therefore, in the second or upper clades, we can only consider comparing the p-values of the subtrees different in the clade. Therefore, when two trees do not have the same number of subtrees in the second or upper clade, it may not be possible to compare their p-values such as in the second clade in this example. In fact, in Step 3 of Procedure 1, we mention that the trees satisfying  $R(1, k)$  could be desired trees if we allow the selection of at least two trees. Therefore, only considering the  $R(1, k)$  criterion and allowing the selection of more than one tree might be an easy way to implement this method.

In this example, we consider 10  $k$  values in the range of (0, 0.1). In this case, we can observe the results for different  $k$  values and make a more objective decision to select trees. For different  $k$  values, the calculation of the  $R(i, k)$  values only requires counting the ratio of p-values less than  $k$ . It does not have much calculation loading. If we do not have any preference in selecting  $k$ , comparing the results for different  $k$  could be a good way to select trees.

#### 4. Discussion

It may not be easy to verify which type of phylogenetic tree is valid for analyzing real data. As a result, it is also hard to verify whether the trees selected by the proposed method are valid. Nevertheless, we use another viewpoint to state the advantage of this proposed method. The sequences, HQ613403.1, and KC242784.1 to KC242790.1, of the illustrated example are used to explain the rationality of our method. When we plot the neighbor-joining tree, nine substitution models can be chosen in MEGA 6. Among all models, the topology trees for the K80 model and Tamura 3-parameter model are the same as in Figure 9B and the topology trees for the other models are the same as in Figure 9A. For the maximum likelihood trees, the topology trees for all models are also the same as in Figure 9A. Therefore, we can say that the topology tree in Figure 9A is more suitable for these sequences.



**Figure 9.** The neighbor-joining trees for the subgroup of ebolavirus sequences. Yellow color: difference. (A) Other models exclude K80 model and the Tamura 3-parameters model; (B) K80 model and the Tamura 3-parameters model.

Note that Figure 9A is the topology tree plotted by the substitution models: p-distance, JC69 model, Tajima Nei model, TN93 model, maximum composite likelihood model, and LogDet model. Figure 9B is the topology tree plotted by the K80 model and the Tamura 3-parameters model.

Now we follow our method to select the tree. Then we need to compare the first clades for these trees. Because the first clades are all the same for these trees, we cannot find a suitable tree from the first clades. Hence, we further consider the second clades for these two trees. Figure 10 shows the second clades for them. Tables 5 and 6 show the p-values for testing the nucleotide substitution number between sequences in the second clade for the neighbor-joining tree based on the JC69 model and the K80 model, respectively. The number of p-values less than the threshold  $k$  in the second clades for each tree is shown in Table 7. We can find that the tree constructed based on the JC69 model has the minimum number of p-values less than  $k$ . Then, according to our method, we also choose Figure 9A to be the suitable tree for these sequences. Therefore, our method is reasonable for selecting a suitable tree.



**Figure 10.** (A) The second clade for the tree in Figure 9A; (B) The second clade for the tree in Figure 9B.

**Table 5.** The p-values for testing the nucleotide substitution number between sequences in the second clade for the tree in Figure 9A.

	HQ613403.1   Zaire	KC242784.1   Zaire
KC242785.1   Zaire	0.012677	0.002341
KC242790.1   Zaire	0.007156	0.001351

**Table 6.** The p-values for testing the nucleotide substitution number between sequences in the second clade for the tree in Figure 9B.

	KC242786.1   Zaire	KC242789.1   Zaire
KC242785.1   Zaire	0.007156	0.004078
KC242790.1   Zaire	0.004078	0.002341

**Table 7.** The numbers of  $p$ -values less than  $k$  in the second clade for the trees in Figure 9. The difference between these two models is marked by the red color in the JC69 model.

		$k$									
Substitution Model		0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
Neighbor-joining	JC69 model	$\frac{3}{4}$	$\frac{4}{4}$								
	K80 model	$\frac{4}{4}$									

## 5. Conclusions

There are many phylogenetic tree construction methods established in the literature such as the maximum likelihood, neighbor-joining, and UPGMA approaches. The topologies of different trees are not exactly the same. Even for the same method, the topologies of trees are different when they embed different nucleotide substitution models. In this study, we propose a methodology to choose a suitable tree. This methodology uses hypothesis testing to test the nucleotide substitution number between sequences and compute their  $p$ -values. An Ebolavirus example is used to illustrate this methodology. A subgroup of Ebolavirus sequences is also used to verify the proposed method. The result shows that the proposed method is a useful approach to choosing a suitable tree.

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