An explicit Poisson-Kolmogorov-Smirnov test for the molecular clock in phylogenies

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Abstract

Divergence dates estimates are central to understand evolutionary processes and depend, in the case of molecular phylogenies, on tests for the molecular clock. Testing for global and local clocks generally compare a clock-constrained tree versus a non-clock tree (e.g. the likelihood ratio test). These tests verify the evolutionary rate homogeneity among taxa and usually employ the chi-square test for rejection/acceptance of the “clock-like” phylogeny. The paradox is that the molecular clock hypothesis, as proposed, is a Poisson process, and therefore, non-homogeneous. Here we propose a method for testing the molecular clock in phylogenies that is built upon the assumption of Poisson stochastic process that accommodates rate heterogeneity and is based on ensembles of trees inferred by the Bayesian method. The observed distribution of branch lengths (number of substitutions) is obtained from the ensemble of post burn-in Bayesian search. The parameter $\lambda$ of the expected Poisson distribution is given by the average branch length of this ensemble. The goodness-of-fit test is performed using a modified Kolmogorov-Smirnov test for Poisson distributions. The method here introduced uses a large number of statistically equivalent phylogenies to obtain the observed distribution. This circumvents problems of small sample size (lack of power and lack of information), because the power of the test is asymptotic to unity. Also, the observed distribution obtained is very robust in the sense that for a sufficient number of trees (700) the empirical distribution stabilizes. Therefore, the estimated parameter $\lambda$, used to define the expected distribution, is essentially independent of sample size.

Keywords: Molecular clock, divergence estimation, molecular phylogenies, phylogenetic trees, evolution, Bayesian inference, Poisson process.
Introduction

The molecular clock hypothesis postulates that for a given informational macromolecule (DNA or protein sequence) the evolution rate is approximately constant over time in all evolutionary lines of descent. This implies that if genetic divergence accumulates in a clock-like manner, that is, an approximately constant number of mutations are accumulated per unit time, then, time scales can be determined for important evolutionary events, even in the absence of fossil evidence. Moreover, the degree of variation of the rate between the lineages could shed light on the mechanisms of molecular evolution.

If the substitution rate is constant between lineages, then the distances between species should be ultra-metric, i.e. all external nodes of a phylogenetic tree should be of the same size starting from the root. In (Zuckerkandl and Pauling 1962; Zuckerkandl and Pauling 1965a; Zuckerkandl and Pauling 1965b) the authors suggested that the substitution process is approximately Poisson. If substitutions follow a Poisson process then the average number of substitutions, and its variance, in different lineages on the same amount of time should be equal.

Notwithstanding the great impact of the molecular clock in evolutionary biology, as comparative molecular data have been accumulating over the past decades, no prediction has been proven to be satisfactory; the dispersion index (the ratio of the variance to the mean value) of the number of substitutions is generally greater than unit, suggesting that the substitution process is over-dispersed. Furthermore, it was observed that the substitution rate usually display variation along the lineages, a fact that has created great controversy related to its use in dating procedures (Swofford et al. 1996). The index of dispersion (the ratio of the variance to the mean of substitutions) has been proposed as an estimator to test the molecular clock. The rationale is that when the number of substitutions follows a Poisson low the index of dispersion equals one. The problems with this type of test have been extensively discussed (Goldman 1994; Nielsen 1997) and since then this approach has been dismissed.

When the neutral theory of molecular evolution was proposed (Kimura 1968; King and Jukes 1969), the observed clock-like behavior of molecular evolution was advocated as one of the strongest evidences supporting the theory (Kimura and Ohta 1971). However, the
reliability of the clock and its implications for the mechanism of molecular evolution were a focus of immediate controversy, entwined in the neutralist–selectionist debate. The controversy surrounding the neutral theory has generated a rich body of population genetics theory and analytical tools. For instance, in the strict neutral model the dynamics depends on the neutral mutation rate alone, however one may expect most sites in a functional protein to be constrained during most of the evolutionary time. This observation motivated the introduction of doubly stochastic Poisson process, or Cox process as a model for the substitution process, implying that positive selection, if it occurs, should affect only a few sites and occur in an episodic fashion (Gillespie 1984, 1986a, 1986b).

Despite the observation that the molecular clock hypothesis does not fully explain the substitution process, it still remains a promising concept and a powerful analytical tool in evolutionary biology. Therefore, testing the molecular clock in phylogenetic trees is an essential task. The problem is that if one assumes that the substitution process is Poisson then there is no homogeneity in the distribution of substitutions along a lineage. In fact, a Poisson process is as heterogeneous as it is possible, since it is often called a completely random process, in the sense that it distributes dots “at random” over a half-infinite line.

**Results and Discussion**

In this work we advance the view that if a phylogenetic tree is clock-like, then the distribution of the branch lengths a phylogenetic tree, when measured as the number of substitutions, follows a Poisson law. Based on this fact we develop a procedure for testing the molecular clock in phylogenetic trees where the inference is made through a non-parametric goodness-of-fit test.

The method proposed here introduces two novelties. The first novelty of the method presented here is that it is bases on an ensemble of trees, instead of using only one single consensus tree – this is quite natural from the point of view of the Bayesian framework. Indeed, the Bayesian inference gives a posterior distribution over the set of phylogenetic trees, in the form of an ensemble of representative trees. The second novelty is the use of a non-parametric goodness-of-fit test based on the *classical Kolmogorov-Smirnov test* (Kolmogorov 1933; Smirnov 1948; Kolmogorov 1992), with appropriate modifications to
account for a Poisson distribution with unknown parameter. We will refer to this procedure as the Poisson-Kolmogorov-Smirnov test.

Starting with an ensemble of phylogenetic trees obtained by Bayesian inference from a set of aligned sequences one extracts an ensemble of branch lengths. Each branch length value is converted into a non-negative integer, the number of substitutions, by multiplying the branch length by the size of the alignment and rounding the value. From the ensemble of branch lengths one may compute the empirical cumulative distribution of the observed data given by

\[ F_o(x) = \frac{\text{number of elements in the sample} \leq x}{\text{sample size}} \]  

where \( x \) is a positive real number. The empirical cumulative distribution \( F_o(x) \) is the input data for a nonparametric goodness-of-fit test.

The Kolmogorov-Smirnov (KS) test is performed in the following way. Given the the empirical cumulative distribution \( F_o(x) \) of the observed data and the cumulative distribution function \( F_e(x) \) of the expected distribution, the test statistic is \( D_{KS} = \sup |F_e(x) - F_o(x)| \), which is a measure of distance between the two distributions, and the null hypothesis (\( H_0: F_e(x) = F_o(x) \)) is rejected if \( D_{KS} \) exceeds a critical value \( D_\alpha \) for a fixed significance level \( \alpha \). In the standard KS test, the distribution of \( D_{KS} \) does not depend on the distribution \( F_e(x) \) and is given by an explicit formula – tabulated critical values have been available since (Smirnov 1948). Moreover, the test has power (sensitivity) tending to 1 as the sample size tends to infinity.

However, the universality of the distribution of \( D_{KS} \) comes at a price: (i) the test only applies to continuous distributions and (ii) the parameters of the expected distribution can’t be estimated from the data – in fact, these two conditions are necessary to show that the distribution of \( D_{KS} \) is independent of the expected distribution. This seems to be a serious objection for the use of the KS test in practical applications. Indeed, the use of the tables associated with standard KS test when one of the conditions (i) or (ii) are not satisfied results in conservative \( p \)-values, in the sense that the probability of type I error is smaller than that given by the standard table (Noether 1963).
Nevertheless, it is possible to circumvent these limitations and modify the KS test to the case of discrete distributions with estimated parameters (Arnold and Emerson 2011). The formula of the test statistic remains unchanged, but its distribution is much more difficult to obtain; unlike the continuous case, it depends on the expected distribution and the estimated parameter. This has prevented the dissemination of the method in biology and other fields. Nowadays, with the advent of fast and cheap computers, p-values and critical values of modified KS tests can be easily computed.

In our particular case, we need a procedure for testing the null hypothesis $H_0$: “$F_o(x)$ is Poisson($\lambda$)” against the alternative hypothesis $H_1$: “$F_o(x)$ is not Poisson($\lambda$)” based on estimating the Poisson parameter $\lambda$ as the mean value of the observed data. The test is performed by calculating the statistic

$$D_{PKS} = \sup |F_o(x) - P(x, \lambda)|,$$

where $P(x, \lambda)$ is the cumulative distribution function for the Poisson distribution with parameter $\lambda$, defined for all positive real values $x$ with $k \leq x < k+1$ for all integral values $k = 0, 1, 2, \ldots, \infty$, as

$$P(x, \lambda) = e^{-\lambda} \left( 1 + \lambda + \frac{\lambda^2}{2!} + \ldots + \frac{\lambda^k}{k!} \right).$$

The null hypothesis $H_0$ is rejected if $D_{PKS}$ exceeds the critical value $D_\alpha$ for a fixed significance level $\alpha$. The value $D_\alpha$ must be obtained from the distribution of the statistic $D_{PKS}$ which depends on kind the distribution $P(x, \lambda)$ and the parameter $\lambda$.

Campbell and Oprian (Campbell and Oprian 1979) developed an approximate KS goodness-of-fit test for the Poisson distribution with unknown parameter. One can eliminate the need for tables by doing a bootstrap KS test as described in (Conover 1972; Henze 1996). However, neither the tables nor the bootstrap KS test are exact and simulations are required to obtain appropriate critical values. More recently, (Frey 2011) developed an exact KS goodness-of-fit test for the Poisson distribution with unknown parameter, together with a new algorithm for the computation of exact $p$-values and exact critical values.
The test is performed in the following way. Generate two ensembles of phylogenetic trees for the same data: one clock-constrained and another non-clock. After the burn-in, the log-likelihood scores should stabilize, and thus all the trees in the ensemble are statistically equivalent. Apply the test to both ensembles. It is expected that the null hypothesis will not be rejected when the test is applied to the clock-constrained ensemble. If the null hypothesis is not rejected when the test is applied to the unconstrained ensemble, as well, then one may conclude that the molecular clock holds for the data that generated both ensembles.

We have applied the test proposed here to the in vitro evolution dataset of SSU rDNA sequences (Sanson et al. 2002). This dataset contains sequences that are neutrally evolving, in other words, without selection where substitution accumulation is by a stochastic process. The alignment was made with CLUSTALW (Larkin et al. 2007) and the phylogenies were inferred using MRBAYES (Ronquist and Huelsenbeck 2003). Three ensembles of phylogenetic trees were generated using the general time-reversible (GTR) model: the clock-like trees, rooted, the non-clock trees rooted and the non-clock trees unrooted (Table 1).

We have found that the test does not reject the null hypothesis for the clock-like ensemble with 95% significance, showing, as expected, that the distribution of branch lengths follows a Poisson law (fig. 1a and Table 1). The test also does not reject the Poisson null hypothesis for the Non-clock rooted ensemble (fig. 2b and Table 1). However, when applied to the non-clock unrooted ensemble the test rejected the null hypothesis with 95% of significance (fig. 1c and Table 1). Note that the lower is the significance level $\alpha$, the lower is the critical value $D_\alpha$ – thus $\alpha$ may be seen as a stringency criterion for the molecular clock. When the test is applied to an ensemble representing 50% of the trees, the PKS test results still holds (Table 2).

The test can also be implemented by directly comparing the clock ensemble versus the non-clock ensemble without fitting the Poisson. In this case (fig. 2) the two distributions are compared by the Kolmogorov-Smirnov test using the algorithm of (Arnold and Emerson 2011) implemented in the software R (R Core Team 2015) to compute approximate p-values (Table 3). In this case the non-clock rooted tree does not reject the molecular clock while the non-clock unrooted ensemble rejects it, because of the differences in the distributions (fig. 2).
Several statistical tests for the molecular clock have been proposed, the most prominent being the **likelihood ratio test** (Felsenstein 1988). Under the clock hypothesis \((H_0)\), there are \(k-1\) parameters corresponding to the ages of the \(k-1\) internal nodes on a rooted tree with \(k\) species. The more general non-clock hypothesis \((H_1)\) allows every branch to have its own rate. Because time and rate are confounded, there are \(2k-3\) free parameters, corresponding to the branch lengths in the unrooted tree. The clock model is equivalent to the non-clock model by applying \(k-2\) equality constraints. If \(l_0\) and \(l_1\) are the log-likelihood values under clock and non-clock models, respectively then \(2\Delta l = 2(l_1 - l_0)\) is compared with the \(\chi^2\) distribution with \(k-2\) degrees of freedom to decide whether the clock hypothesis is rejected.

The likelihood ratio test has some limitations. First, it should be noted that it does not examine whether the rate is constant over time. In fact, what is tested is the weaker hypothesis that all tips of the tree are equidistant from the root, with distance measured by the number of substitutions. Thus, if the evolutionary rate has been accelerating (or decelerating) over time in all lineages, the tree will look clock-like, although the rate is not constant. Second, the test cannot distinguish a constant rate from an average variable rate within a lineage, although the latter may be a more sensible explanation than the former when the clock is rejected and the rate is variable across lineages. Finally, failure to reject the clock hypothesis may simply be due to lack of information in the data or lack of power of the test.

The method introduced here avoids some of these drawbacks. The use of an ensemble of trees, instead of one consensus tree, has two main advantages. First, the observed distribution obtained is very robust in the sense that for a sufficiently many trees (approximately 700 seems to be enough) the empirical distribution stabilizes. Therefore, the estimated parameter \(\lambda\) used to define the expected distribution is essentially independent of the sample size (fig. 3). Second, the use of a huge number of statistically equivalent phylogenetic trees to obtain the observed distribution allows on to bypass the problems of small sample size (lack of power and lack of information), since the power of the test is asymptotic to unity.
Future perspectives include: (i) use (Frey 2011) algorithm to have a full range of critical values and exact $p$-values; (ii) capability to test local clock hypothesis in different lineages by analysis of subsets and subtrees from the same alignment and (iii) ensembles of trees generated by other programs; (iv) use of more modern tests of the same type, e.g., the Cramér-von Misses (CM) test and the Anderson-Darling (AD) test.

Materials and Methods

The PKS method, here described, is implemented using the PYTHON (version 2.7) programming language (van Rossum 1995), with libraries Numpy, Pylab, Matplotlib and Tkinter. The source code is available as Supplementary Data and at GitHub: https://github.com/FernandoMarcon/PKS_Test.git

The program can directly import the results generated by MRBAYES (Ronquist and Huelsenbeck 2003) and extract an ensemble of trees according to the user choice of size (by choosing a percentage of the total set of trees in the input file). The trees in the ensemble are sorted according to their log-likelihood scores from the lowest to the highest. The Poisson-Kolmogorov-Smirnov (PKS) test is implemented using the tables of (Campbell and Oprian 1979) and supplemented and refined by the tables in (Papadopoulos and Qiao 2003) for the computation of critical values. The user may choose one of the three possible significance levels 0.90, 0.95, 0.99. In (Campbell and Oprian 1979) there are five tables corresponding to the cases $\lambda \in (0, 1]$, $\lambda \in (1, 2]$, $\lambda \in (2, 3]$, $\lambda \in (3, 5]$, and $\lambda \in (5, 10]$, with $\alpha = 0.01, 0.05, 0.10, 0.15, 0.20$ – we have omitted the last two options, since they are rarely used. The program outputs the following information: (i) the mean value and the variance of the log-likelihood scores of the ensemble of trees; (ii) the mean and the variance of the observed distribution of branch lengths, measured in number of substitutions; (iii) the PKS statistic $D_{PKS}$ and the critical value $D_\alpha$ for the corresponding significance level. Finally, there is the option to plot the curves corresponding to the empirical and expected probability distribution functions, which are much better to visualize than the cumulative distribution functions.
Acknowledgements
This work was supported by a FAPESP (2013/07838-0) grant to M.R.S.B. and CNPq (Brazil) productivity fellowships to F.A. and M.R.S.B.

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Table 1. A PKS test with the ensemble extracted from a set of about 2000 trees.

| Parameters                  | Clock (rooted) | Non-clock (rooted) | Non-clock (unrooted) |
|-----------------------------|----------------|--------------------|----------------------|
| **Number of trees**         | 1990           | 2000               | 2000                 |
| **Ensemble size (100%)**    | 1989           | 2000               | 2000                 |
| **Mean (λ)**                | 5.87           | 6.09               | 7.02                 |
| **Variance**                | 5.37           | 12.06              | 17.01                |
| **Log-likelihood mean**     | -4385.95       | -4380.95           | -4376.06             |
| **Log-likelihood variance** | 5.47           | 7.01               | 3.42                 |
| $D_{PKS} = \sup |F_o(x) - P(x,\lambda)|$ | 0.022       | 0.111              | 0.138                |
| $D_\alpha (1 - \alpha = 0.95)$ | 0.134       | 0.134              | 0.134                |
Table 2. A PKS test with a 50% ensemble extracted from a set of about 2000 trees.

| Parameters                          | Clock (rooted) | Non-clock (rooted) | Non-clock (unrooted) |
|-------------------------------------|----------------|--------------------|----------------------|
| Number of trees                     | 1990           | 2000               | 2000                 |
| Ensemble size (50%)                 | 1000           | 1000               | 1000                 |
| Mean ($\lambda$)                    | 5.87           | 6.08               | 7.02                 |
| Variance                            | 5.34           | 12.55              | 16.97                |
| Log-likelihood mean                 | -4385.95       | -4380.95           | -4376.06             |
| Log-likelihood variance             | 5.47           | 7.01               | 3.42                 |
| $D_{PKS} = \sup |F_o(x) - P(x, \lambda)|$ | 0.022          | 0.111              | 0.138                |
| $D_{\alpha} (1-\alpha = 0.95)$     | 0.134          | 0.134              | 0.134                |
Table 3. A Kolmogorov-Smirnov test comparing the two ensemble distributions with a 100% ensemble extracted from a set of about 2000 trees.

| Parameters                        | Clock (rooted) | Non-clock (rooted) | Non-clock (unrooted) |
|----------------------------------|----------------|--------------------|----------------------|
| Number of trees                  | 1990           | 2000               | 2000                 |
| Ensemble size (100%)             | 1989           | 2000               | 2000                 |
| Mean ($\lambda$)                 | 5.87           | 6.09               | 7.02                 |
| Variance                         | 5.37           | 12.06              | 17.01                |
| Log-likelihood mean              | -4385.95       | -4380.95           | -4376.06             |
| Log-likelihood variance          | 5.47           | 7.01               | 3.42                 |
| $D_{KS} = \sup |F_{clock}(x) - F_{non-clock}(x)|$ | -              | 0.11               | 0.17                 |
| $p$-value (R with DGOF)          | -              | 0.5                | 0.1                  |
Figure Legends

Figure 1. Expected (Poisson) and observed cumulative distribution functions of the clock ensemble (a), non-clock rooted ensemble (b) and non-clock unrooted ensemble (c). Vertical dotted lines indicate the $\lambda$ parameter (mean) of the Poisson distribution. The results of the PKS tests are summarized in Table 1.

Figure 2. Direct comparison of observed cumulative distribution functions of the clock ensemble versus non-clock rooted ensemble (a) and clock ensemble versus the non-clock unrooted ensemble (b). The results of the Kolmogorov-Smirnov tests are summarized in Table 3.

Figure 3. Mean value of the branch length as a function of the number of trees in the ensemble. The average becomes more stable as the number of trees increases.
Fig. 1
Fig. 2

a) Clock vs Non-clock (rooted)

b) Clock vs Non-clock (unrooted)
Fig. 3

A line graph showing the mean response against the percentage of trees.

Y-axis: Mean
X-axis: % trees

The graph indicates a slight increase in the mean value as the percentage of trees increases.