Nonparametric Estimation of Phylogenetic Tree Distributions

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Abstract.—While the majority of gene histories found in a clade of organisms are expected to be generated by a common process (e.g. the coalescent process), it is well-known that numerous other coexisting processes (e.g. horizontal gene transfers, gene duplication and subsequent neofunctionalization) will cause some genes to exhibit a history quite distinct from those of the majority of genes. Such “outlying” gene trees are considered to be biologically interesting and identifying these genes has become an important problem in phylogenetics. In this paper we propose and implement, \texttt{kdetrees}, a nonparametric method of estimating distributions of phylogenetic trees, with the goal of identifying trees which are significantly different from the rest of the trees in the sample.

Our approach mimics the common statistical technique of kernel density estimation, using tree distances to define kernels. In contrast to parametric models, such as the coalescent, nonparametric approaches avoid the problem of model mis-specification, which
leads to potentially unreliable results. Our method demonstrated significantly faster computational time, while suffering only a small penalty to classification accuracy, as compared to a recently published method.

We also applied our method to a data set of Apicomplexa genes, as well as a set of Epichloë genes, fungi symbiotic with grasses. In the case of the Apicomplexa, \texttt{kdetrees} identified several unreliable sequences which had escaped previous detection, as well as gene independently reported as a probably case of horizontal gene transfer. Our method for estimating tree distributions is implemented as the \texttt{R} package, \texttt{kdetrees}, and is available for download from CRAN. (Keywords: phylogenetics, nonparametric, gene trees, \texttt{R} package)
A central problem in systematic biology is the reconstruction of populations and species from numerous gene trees with varying levels of discordance (Brito and Edwards 2009; Edwards 2009). Although there is a well-established understanding that discordant phylogenetic relationships will exist among independent gene trees drawn from a common species tree (Maddison 1997; Pamilo and Nei 1988; Takahata 1989), phylogenetic studies have only recently begun to shift away from single-gene and concatenated-gene estimates of phylogeny in favor of multi-locus methods (Carling and Brumfield 2008). These newer approaches focus on the role of genetic drift in producing patterns of incomplete lineage sorting and gene tree/species tree discordance, largely using coalescent theory (Degnan and Salter 2005; Rosenberg 2002, 2003). These theoretical developments have been used to reconstruct species trees from samples of estimated gene trees (Carstens and Knowles 2007; Edwards et al. 2007; Maddison and Knowles 2006; Mossel and Roch 2010; RoyChoudhury et al. 2008).

Detecting concordance among gene trees is also a topic of interest. For example, Ané et al. (2007) developed a Bayesian method to estimate concordance among gene trees using molecular data from multiple loci. The method can produce estimated gene trees as well as an estimate of the proportion of the genome support a particular clade. However, a priori assumptions must be made about the degree and structure of concordance present in the gene trees.

Although there is a tremendous amount of ongoing effort to develop better parametric models for gene tree distributions, the parametric framework has inherent limitations. While a parametric method typically makes the most efficient use of a given data set when the model is specified correctly, they achieve this efficiency by assuming that the true distribution of gene trees is one of a relatively small class of distributions. This
can lead to erroneous inferences when the true distribution does not resemble any of the models in the proposed class. Given that many questions remain about the proper way to incorporate a number of important processes into a parametric model (e.g., geographic barriers to migration, or a population bottleneck), the problem of model mis-specification is very real. Nonparametric methods avoid the majority of these modeling issues, enabling unbiased estimation for a much larger class of true tree distributions.

Numerous processes can reduce the correlation among gene trees. Negative or balancing selection on a particular locus is expected to increase the probability that ancestral gene copies are maintained through speciation events \cite{Takahata and Nei 1990}. Horizontal transfer introduces divergent gene copies into a different species through a vector, or shuffles gene copies among species via hybridization \cite{Maddison 1997}. The correlation may also be reduced by naive sampling of loci for analysis. For example, paralogous gene copies will result in a gene tree that conflates gene duplication with speciation. Similarly, sampled sequence data that span one or more recombination events will yield “gene trees” that are a hybrids of two or more genealogical histories \cite{Posada and Crandall 2002}. These non-coalescent processes can strongly influence phylogenetic inference \cite{Edwards 2009, Martin and Burg 2002, Posada and Crandall 2002}. In addition, \cite{Rivera et al. 1998} showed that analyses of complete genomes indicated a massive prokaryotic gene transfer (or transfers) preceding the formation of the eukaryotic cell, arguing that there is significant genomic evidence for more than one distinct class of genes. These examples suggest that the distribution of eukaryotic gene trees may be more accurately modeled as a mixture of a number of more fundamental distributions.

In this paper, we focus on the problem of identifying significant discordance among gene trees, as well as estimating the distribution of gene trees as a whole. This set of gene
trees is assumed to consist mostly of “typical” (or “non-outlier”) gene trees, which are assumed to be independently sampled from some distribution $f$. For example, gene trees which have evolved neutrally under a coalescent process. In addition, there are a smaller number of “outlier” gene trees which are sampled from a very different distribution $f'$. These genes are assumed to arise from less common evolutionary processes; for example, neofunctionalization, horizontal gene transfer, or periods of rapid molecular evolution. In addition, more mundane errors—such as incorrect sequencing, alignment, tree reconstruction, or annotation—can also produce outlier trees in a data set (Horner and Pesole 2004). Our method produces a nonparametric estimate of the distribution $f$ and also attempts to identify potential outlier gene trees which are probably not generated by $f$. Trees identified as outliers can then be inspected more closely for biologically interesting properties. In particular, identifying and removing outliers that violate model assumptions can improve the accuracy of inferences made from a collection of gene trees (e.g. (Disotell and Raaum 2004; Edwards 2009; Martin and Burg 2002; Posada and Crandall 2002)).

Related Work

The method presented in this paper is not a statistical method for hypothesis testing, but rather for “discovering possible outliers” present in a given collection of orthologous genes. However, there has been significant work devoted to the development of statistical methods for testing hypotheses of discordance between the trees in a collection. The reviewed methods in Poptsova (2009) are the following: (i) likelihood-based tests of tree topologies (Kishino-Hasegawa (KH) (Kishino and Hasegawa 1989), Shimodaira-Hasegawa (SH) (Shimodaira and Hasegawa 1999), Approximately Unbiased (AU) tests (Shimodaira 2002)); (ii) tree distance methods (Robinson–Foulds (RF) and subtree pruning and regrafting
(SPR) distances); and (iii) genome spectral approaches (bipartition \cite{Lockhart1995} and quartet decomposition analysis \cite{Piaggio2004}).

The likelihood-based tests of tree topologies and tree distance methods are statistical hypothesis tests that detect significant incongruence between trees, i.e., they are testing the following hypotheses:

\[ H_0 : \text{Given trees are congruent.} \]
\[ H_1 : \text{Given trees are incongruent.} \]

The distinction between likelihood and distance based methods is in how they calculate the p-value of these hypotheses. The likelihood-based tests compare each gene tree with a species/reference tree using a likelihood value, to see if the incongruence is “statistically significant.” These methods are also known as partition likelihood support (PLS) \cite{Lee2003}. Tree distance methods estimate the p-value of the hypotheses above by computing a distance between a reference tree and each gene tree. \cite{Holmes2005} describes a framework for statistical hypothesis testing on trees based on tree distances using distributions of phylogenetic trees (e.g. a posterior distribution or bootstrap resampling).

Holmes also presents a statistical method to compare two sets of bootstrap sampling distributions, using the mean and variance of each distribution \cite[Section 4.4.1]{Holmes2005}. A statistical nonparametric method for detecting significant discordance between two sets of trees via the supporting vector machines (SVMs) was introduced by \cite{Haws2012}. This is a nonparametric method for statistical testing of the hypotheses:

\[ H_0 : \text{Two sets of trees are drawn from the same distribution.} \]
\[ H_1 : \text{Two sets of trees are not drawn from the same distribution.} \]

While likelihood-based tests assume that the species tree is known, genome spectral
approaches do not use such a reference tree. Genome spectral methods summarize a set of
gene trees with phylogenetic spectra (frequencies), such as splits or quartets. These
frequencies can be used to approximate the distribution of gene trees, instead of producing
a summarizing tree. Outlier trees can be identified by looking for trees whose highly
supported features disagree with prevalent features in the spectra (Nepusz et al. 2010).

A non-statistical approach for summarizing collections of gene trees is presented by
Nye (2008). Treating each gene tree as a leaf node, a “meta-tree” is constructed where
nodes correspond to phylogenetic trees; distances between nodes of the meta-tree
correspond to distances between phylogenetic trees, and internal nodes correspond to gene
trees with various branches collapsed. When using the RF distance, the nonparametric
method proposed in this paper can be viewed as a numerical summarization of the
meta-tree in (Nye 2008).

Recently, de Vienne et al. (2012) developed a statistical nonparametric method to
detect outlier trees from the set of gene trees. They first convert gene trees into vectors in
a multi-dimensional Euclidean space and then apply Multiple Co-inertia analysis—an
extension of Principal Coordinate analysis (PCO)—directly to these vectorized gene trees.
Their method, Phylo-MCOA, also detects outlier species, those whose position varies widely
from tree to tree. Included in our results are simulation studies comparing our
non-parametric method with Phylo-MCOA.

Overview of Our Approach

Let $\mathcal{T}_n$ denote the set of all tree topologies on $n$ taxa (which we call tree space). We consider
the trees to be unrooted, but rooted trees can be treated similarly. Our main object of
study is a sample, $\{T_i\}_{i=1}^N$, of $N$ trees (gene trees) mostly drawn from a distribution $f$ on
\( T_n \). If \( n \) is large enough that \( |T_n| \gg N \) then many tree topologies in the sample may have low empirical frequency. In this case, \( f \) cannot be estimated well by assigning \( \hat{f}(T) \) to be the empirical frequency of \( T \) in the sample. On the other hand, if \( f \) corresponds to a model such as coalescent, it is reasonable to expect that topologies “close” to many observed trees will have a higher likelihood than topologies “far away” from the observed trees.

*Kernel density estimation* is a nonparametric technique to estimate a distribution that generated a sample, by leveraging the fact that points close to sample points tend to have higher likelihood than distant outlier points (under adequate assumptions on the distribution, namely, the distribution is square-integrable \cite{Meloche1990}). Kernel density estimation can be viewed as a refined version of histogram-based estimation of a density. As the term *density* suggests, kernel density estimation is typically formulated for continuous variables over \( \mathbb{R}^d \). However, similar methods can also be devised to estimate distributions over a finite set such as tree space. A key ingredient is the ability to measure similarity between trees. Fortunately, research in phylogenetics has produced several classical distances on tree space, such as the dissimilarity map distance \cite{Buneman1971}, the topological dissimilarity distance measure \cite{Steel1993}, the Robinson–Foulds distance \cite{Robinson1981}, and the quartet distance \cite{Estabrook1985}. More recently \cite{Billera2001} introduced the notion of geodesic distances. \cite{Owen2011} showed that there is an efficient algorithm for computing this distance in \( O(n^3) \), where \( n \) is the number of taxa.

Our method uses existing tree distances to estimate a tree distribution by mimicking kernel density estimation. Our main goal is to identify regions of \( T_n \) which have high probability, as well as observed trees with markedly low estimated probability. These low-probability trees are potentially outlier trees; i.e., trees having evolutionary histories
unlikely to have arisen from the same model that generated the non-outlier trees. Our approach is nonparametric, which makes it quite general, and avoids problematic issues such as model design and selection that one encounters when using a parametric model (such as the coalescent). Unfortunately, using a small sample to learn an arbitrary distribution on tree space is inherently difficult, especially as the dimension of $T_n$ grows, and we do not expect to learn the tree distribution with high accuracy for every tree topology. However, estimates of the density in regions where the probability is high can be quite good.

Our method identifies potential outliers in a set of trees by comparing the values of the non-normalized density estimates (which we call “tree scores”) of the trees. An unusually low score indicates that a tree is relatively distant from the other trees in the sample. We implement a simple classification scheme which is based on the interquartile range (IQR) of the density estimates, as is commonly done when creating box-and-whisker plots.

**Materials and Methods**

Given an independent and identically distributed sample of trees $T_1, \ldots, T_N$, we propose a nonparametric estimator of the distribution that generated the sample with the form

$$\hat{f}(T) \propto \frac{1}{N} \sum_{i=1}^{N} k(T, T_i).$$

Here $k$, the kernel function, is a non-negative function defined on pairs of trees which measures how “similar” two trees are. For our approach, we do not require $k$ to be a kernel in a strict statistical sense.
In \texttt{kdetrees} we have implemented a kernel of the form,

\[ k(T, T_i) = \frac{1}{h_i} \exp\left( -\left( \frac{d(T, T_i)}{h_i} \right)^\delta \right), \]

where \( d \) is a distance metric on trees, \( \delta > 0 \) is a “shape” parameter, and the \( h_i > 0 \) are a set of “bandwidth” parameters that control how tightly each contribution \( k(T, T_i) \) will be centered on \( T_i \). Allowing the bandwidth to vary with the sample points, \( T_i \), is called an \textit{adaptive bandwidth} method, alternatively the bandwidth can be set to a constant value for all \( T_i \).

In general, we can remove the symmetry and triangle inequality requirements for \( d \), and it is possible that the sum over tree space, \( \sum_{T \in \mathcal{T}} k(T, T') \), will vary with \( T' \). Ideally, we would remedy this issue by normalizing \( k(\cdot, T') \) so that \( \sum_{T \in \mathcal{T}} k(T, T') = 1 \). (This is the case most analogous to kernel density estimation.) However, for reasonable choices of \( d \), \( \sum_T k(T, T') \) is not expected to vary significantly for most \( T' \), so we ignore this issue rather than trying to compute or estimate each \( \sum_{T \in \mathcal{T}} k(T, T_i) \).

Since the ultimate goal is to detect outlier trees, \( T_j \), which are not actually drawn from the true distribution \( f \), we are most concerned with estimating the density at the observed sample points. In this context, it makes sense to use a “leave-one-out” estimator which excludes the contribution of the point in question from the tree score,

\[ \hat{g}(T_j) = \frac{1}{N-1} \sum_{i \neq j} k(T_j, T_i). \]

Assuming the sample is drawn i.i.d. from a distribution \( f \), for fixed \( d \) and \( \delta \), both \( \hat{g}(T) \) and \( \hat{f}(T) \) (once normalized) will converge to \( f \) as \( N \to \infty \), so long as the \( h_i(N) \to 0 \). This result follows immediately from the finiteness of tree space.
Once we have computed the scores, $\{\hat{g}(T_i)\}$, we classify tree $T_j$ as an outlier if $\hat{g}(T_j)$ is less than $Q_1 - \kappa \cdot IQR$. $Q_1$ and $IQR$ are the first quartile and the interquartile range of the set of tree scores, respectively; and $\kappa$ is a classification tuning parameter. The choice of $\kappa$ affects the sensitivity and specificity of the classifier, and is set to 1.5 by default, although the user may supply their own value.

Choice of tree distance.—In our approach, trees can be incorporated into a statistical framework by converting them into a numerical vector format based on a distance matrix or map. These vectorized trees can then be analyzed as points in a multi-dimensional space where the distance between trees increases as they become more dissimilar (Graham and Kennedy 2010; Hillis et al. 2005; Semple and Steel 2003).

For the choice of $d$, we propose distances derived from three different distances on trees: dissimilarity map $d_{\text{dis}}$, topological dissimilarity map $d_{\text{top}}$, and geodesic distance $d_{\text{geo}}$. The dissimilarity map distance measure between two trees is the euclidean distance,

$$d_{\text{dis}}(T', T) = ||v_{\text{dis}}(T) - v_{\text{dis}}(T')||_2,$$

where $v_{\text{dis}}(T) \in \mathbb{R}^{n^2}$ is the vector whose $(i, j)$-th entry is the sum of branch lengths on the path between leaves $i$ and $j$ in $T$. The dissimilarity map distance is studied in Buneman (1971). The topological dissimilarity map distance measure between two trees is the euclidean distance,

$$d_{\text{top}}(T', T) = ||v_{\text{top}}(T) - v_{\text{top}}(T')||_2,$$

where $v_{\text{top}}(T) \in \mathbb{R}^{n^2}$ is the vector whose $(i, j)$-th entry counts the number of edges between leaves $i$ and $j$ in $T$. The topological dissimilarity distance measure, also called the path difference, was studied in Steel and Penny (1993). An example calculation of both $v_{\text{dis}}$ and
Billera et al. (2001) showed that the space of trees with a fixed number of taxa is the union of positive cones in $\mathbb{R}^{n(n-2)}$. Thus, the tree space, also called CAT(0) space, is the set of all tree metrics, and is a subspace of the space of all distance matrices. The geodesic distance $d_{geo}$ is the distance between two trees when paths are constrained within CAT(0) space (note that the space of trees with $n$ taxa is not Euclidean). Owen and Provan (2011) developed the $O(n^3)$ algorithm to compute the geodesic distance $d_{geo}(T, T')$ where $T, T' \in \mathcal{T}_n$.

**Missing taxa.**—It is desirable for phylogenetic analyses to be able to deal with situations with incomplete data. In this case, the most relevant type of missing data is when some gene trees are missing a tip which is present in other trees in the dataset. Our method is capable of handling such a situation if the dissimilarity or topological distance methods are used. In this situation we impute missing tip-to-tip distances in the tree vectors with the median value found in trees containing the missing tip. The geodesic distance method does not employ such a vectorization step, and does not handle missing tips at this moment.

If the trees have node labels which correspond to support for the given split (obtained, for example, by a bootstrap analysis), then the software can accommodate this information by collapsing nodes with support less than a given value. This behavior is disabled by default.

**Estimation of bandwidth.**—The estimator $\hat{g}$ depends crucially on the choice of the bandwidth parameter $h$. We employ a nearest-neighbor approach to estimate an adaptive bandwidth for each sample point. To estimate the bandwidth for a point $T_j$, we use the distance to the $m$-th closest sample point. This approach has the effect of causing the kernels to be concentrated in areas where there is a lot of data, and diffuse in the tails of
the distribution. In the current version of \texttt{kdetrees} \(m\) is defaulted to be 20\% of the sample size, a heuristic value chosen based on simulation results.

Alternatively, the bandwidth can be set to a constant value for all \(T_i\). In order to do this we must find a way to choose an optimal value for the bandwidth \(h\). We experimented with a constant bandwidth chosen by estimating the partition function \(Z_h = \sum_T \hat{g}_h(T)\) using a random sample of trees. However, it seems that we tend to under-estimate the bandwidth \(h\) and the results are not as robust as in the case of the adaptive bandwidth.

\textit{Tuning parameters.}—The the outlier classifier’s sensitivity depends on the choice of a tuning parameter, \(\kappa\). The default value, 1.5, is chosen for hisorical reasons. In our simulations smaller values of \(\kappa\), around 0.75 to 1, often resulted in false positive rates close to 5\%. Creating plots of the tree scores may be helpful in chosing an appropriate value for a given data set.

\textit{Running time.}—The computation time of \texttt{kdetrees} is dominated by the step where pairwise tree distances are calculated. For \(N\) trees, each with \(n\) taxa, this step takes \(O(n^2 N^2)\) operations when using the dissimilarity or topological distances, or \(O(n^3 N^2)\) if using the geodesic distance.

\textit{Simulation}

We conducted a series of simulations comparing the performance of \texttt{kdetrees} and \texttt{Phylo-MCOA}. \texttt{Phylo-MCOA} is a \texttt{R} package and one of the functions in the software is to identify putative outlying genes in a data set. In most of these simulations, the data consisted of coalescent trees generated by the Python library DendroPy (Sukumaran and Holder 2010). Six species trees (Figure 1) were used to contain coalescent gene trees. A
data set consisted of a small number (one, unless otherwise stated) of “outlier” gene trees, together with a larger number (100, unless otherwise stated) of “non-outlier” gene trees which were similar to each other. In the “single” coalescent simulations, the non-outlier trees are all contained within a the top left tree in Figure 1. In the “mixed” coalescent simulations, an equal number of non-outlier genes were sampled from each of the other 5 trees. Figure 2 summarizes the simulation processes. Code and documentation for the simulations is included in the kdetrees software package download (see package vignettes). The Python scripts used to generate the simulated data sets are also included.

Our first simulation investigated the classification characteristics of the methods, producing ROC curves comparing kdetrees and Phylo-MCOA, by varying the classification tuning parameter of each method. In this simulation we set the effective population size of the coalescent process generating the trees to 2000, a value which produced a moderate amount of variance in the generated trees.

A second simulation compares the true positive rates of the methods as the variance of the coalescent trees changes. This simulation was carried out both with the default tuning values, as well as values chosen based on the ROC simulation results to limit the FPR to around 5%.

A third simulation compared the distribution of outlier tree scores to the distribution of non-outlier tree scores. Five hundred coalescent trees were generated within the species tree in the top-left of Figure 1. The effective population size for these trees was also 2000. Scores for the trees in this data set were computed, and an estimate of the distribution of these non-outlier scores produced. To estimate the distribution of non-outlier scores, a single random coalescent tree was appended to the set of trees previously generated, and the score for this new outlier tree was computed. This process
was repeated 1000 times (always using the same set of non-outlier trees) to generate a sample of outlier tree scores and an estimate of this distribution was produced. The simulation process is summarized in Figure 3.

Finally, the running time was measured as the number of trees in the data set was varied. These measurements were made on a 2.3 GHz dual-core MacBook Pro with 16GB of physical memory.

**Apicomplexa Data**

The Apicomplexa data set presented by Kuo et al. (2008) consists of gene trees reconstructed from sequences from the following species: *Babesia bovis* (Bb) (Brayton et al. 2007) (GenBank accession numbers AAXT01000001–AAXT01000013), *Cryptosporidium parvum* (Cp) (Abrahamsen et al. 2004) from CryptoDB.org (Heiges et al. 2006), *Eimeria tenella* (Et) from GeneDB.org (Hertz-Fowler et al. 2004), *Plasmodium falciparum* (Pf) (Gardner et al. 2002) and *Plasmodium vivax* (Pv) from PlasmoDB.org (Bahl et al. 2003), *Theileria annulata* (Ta) (Pain et al. 2005) from GeneDB.org (Hertz-Fowler et al. 2004), and *Toxoplasma gondii* (Tg) from Toxo-DB.org (Gajria et al. 2008). A free-living ciliate, *Tetrahymena thermophila* (Tt) (Eisen et al. 2006), was used as the outgroup. To this set of sequences, we appended the Set8 gene, which has been identified by Kishore et al. (2013) as a probable case of horizontal gene transfer from a higher eukaryote to an ancestor of the Apicomplexa.
**Fungal Data**

Another set of biological sequences to use as a test case was generated from housekeeping genes and a known pair of paralogs in *Epichloë* species and related plant symbionts and parasites in the fungal family Clavicipitaceae. We previously reported sequencing, annotation, and the identification of orthologs in genome of *Epichloë amarillans* strain E57, *E. brachyelytri* E4804, *E. festucae* strains E2368 and F11, *E. glyceriae* E277, *E. poae* E5819, *E. typhina* E8, *Aciculosporium take* MAFF-241224, *Claviceps fusiformis* PRL 1980, *C. paspali* RRC-1481, *C. purpurea* 20.1, *Neotyphodium gansuense* e7080, and *Periglandula ipomoeae* IasaF13 [Schardl et al. 2013]. We compiled the inferred protein sequences for ten housekeeping proteins, namely, [gamma]-actin (ActG), DNA lyase (ApnB), a calmodulin-dependent protein kinase (CpkA), the largest and second largest subunits of RNA polymerase II (rpbA and rpbB), translation elongation factor 1-[alpha] (TefA), [alpha]-tubulin (paralogs TubB and TubC), and [beta]-tubulin (paralogs TubB and TubP). As the expected phylogenetic outlier, we compiled two known paralogous proteins, namely, LolC (which catalyzes synthesis of a loline alkaloid intermediate), and the very closely related O-acetylhomoserine(thiol)-lyase (CysD, which scavenges H2S for synthesis of a methionine intermediate) [Spiering et al. 2005]. Of the 13 fungal strains, three had lolC genes but not cysD, nine had cysD but not lolC, and one (*E. glyceriae* E277) had both genes. Both LolC/CysD datasets had one sequence from each strain, but they differed in containing either LolC or CysD from *E. glyceriae*. 
Results

We present the R software package \texttt{kdetrees} for nonparametric estimation of tree distributions and detection of outlier trees. The software takes a sample of trees, \(T_1, \ldots, T_N\), in Newick format as input, and estimates for each tree a “score” based on a nonparametric estimator of the tree density. The \texttt{kdetrees} package can also attempt to improve the estimator \(\hat{f}\) by applying a greedy approach to iteratively remove potential outlier trees from the computation. The output is a list of scores, which are the density estimates of the observed trees. Plots summarizing the findings can be produced, as well as a list of putative outlier trees.

The \texttt{kdetrees} package is written in R (R Development Core Team 2011), and makes use of some functions in the packages \texttt{distory}, \texttt{ggplot2}, and \texttt{ape} (Chakerian and Holmes 2013; Paradis et al. 2004; Wickham 2009). It has been tested on the Windows, OSX, and Linux platforms, and is released under the General Public License. The software is available for download from CRAN. Source code can be obtained either from CRAN, or from \url{http://github.com/grady/kdetrees/}.

Simulation Results

Our first simulation, presented in Figure 4, produced ROC curves comparing the various methods of outlier identification. We find that the performance of \texttt{kdetrees} and \texttt{Phylo-MCOA} is similar, with \texttt{Phylo-MCOA} having a slightly better curve in the single simulations, and \texttt{kdetrees} in the mixed. Interestingly, the geodesic distance worked better in for the “single” data than the dissimilarity distance, while the relationship is reversed for the “mixed” simulation. These results were almost completely unaffected by changes in
the proportion of outliers in the sample (proportions between 1 to 10% were tested).

The variability of the coalescent trees is determined by the effective population size, the parameter studied in our second simulation. The proportion of the simulated data sets where each method correctly identified an added outlier tree is illustrated in Figure 5. This simulation was run both with default tuning parameters and ones chosen based on the ROC curve simulation results. If optimal tuning parameters are selected, Phylo-MCOA can outperform kdetrees, however, selecting these correctly can be difficult, and kdetrees performs better with the default values selected.

We ran a third simulation studying the difference between the score distributions of outlier trees and non-outlier trees, as the ability of our method to reliably detect outlying trees depends on a tendency by outlier trees to produce scores significantly lower than the scores of non-outlier trees. The results are presented in Figure 6. We found that while there is some overlap between the score distributions, the distribution of scores for outlier trees lies significantly below that of non-outlier trees.

Finally, Figure 7 summarizes the running times of the algorithms as the number of trees in the data set is increased. Here kdetrees vastly outperforms Phylo-MCOA. For a data set consisting of 5000 trees, each with 50 tips, kdetrees completed in about 7.5 minutes, while Phylo-MCOA required slightly over 4 hours. For smaller data sets, of a few hundred trees, kdetrees runs in less than a second, while Phylo-MCOA requires a few minutes.

Application to Biological Data Sets

We applied our method implemented in kdetrees to the Apicomplexa gene data set presented in [Kuo et al. (2008)], to which we appended the Set8 gene, which has been
identified as a probable case of horizontal gene transfer from an animal host to the ancestral apicomplexan [Kishore et al. 2013]. When employing the either the dissimilarity or geodesic map method, our method identified the same set of putative outlier trees. Closer inspection of these trees suggests that these trees correspond to questionable sequence alignments which likely arose either from the inclusion of non-homologous genes in the data set, or from poor sequence annotations, many involving *Eimeria tenella* (Et) sequences.

The list of putative outlier genes selected by kdetrees is presented in Table 1, with additional discussion in Supplemental Table S.1. The first four trees identified as putative outliers are plotted in Supplemental Figure S.2. These trees all contain branches with extremely disproportionate lengths, which lead to their identification as outliers. Although the the Set8 gene was not included in the list of outliers, it fell just short of the threshold needed to be classified as such. Slightly lowering the tuning parameter to $\kappa = 1.3$ (from 1.5) resulted in Set8 being classified as an outlier, and it is the next gene to be identified as such as the classification parameter is lowered.

Since there appeared to be problems with the Et sequence data, we removed these sequences from the dataset and recreated the phylogenetic analysis as in Kuo et al. (2008). With the new set of gene trees, kdetrees identified a different set of outlier trees, and in this case the Set8 gene was selected as the furthest outlying tree.

We also applied kdetrees to a compilation of 11 protein alignments from 13 fungal genomes, where ten of the proteins represented known housekeeping genes, and one alignment included known paralogs, LolC and CysD (Spiering et al. 2005). Because *E. glyceriae* E277 had both lolC and cysD, we ran the analysis on alternative data sets with either LolC or CysD eliminated for that strain. In both analyses, the LolC/CysD tree was identified as one of two outliers, the other being the DNA lyase protein ApnB.
Topologically, the LolC/CysD gene tree differed markedly from the others, which as expected because CysD sequences grouped together in a clade apart from LolC. However, the topology of the ApnB tree was similar to that of other housekeeping genes, suggesting that it had significantly different relative branch lengths.

Running Time

A significant advantage of \texttt{kdetrees} over \texttt{Phylo-MCOA} is a significant improvement in speed, especially with larger data sets. The \texttt{kdetrees} running times appear to be well fitted by a $O(N^2)$ curve, as suggested by the complexity of the algorithm discussed previously, while the \texttt{Phylo-MCOA} times appear to be $O(N^3)$.

Discussion

Our proposed new method designated NEPTD is motivated by the fact that existing methods of phylogenetic analysis and tree comparisons are not adequate for genomic scale phylogenetic analysis, particularly in cases of certain non-canonical evolutionary phenomena. In simulations and applications to biological data, we address particular challenges posed by bioinformatic artifacts, as well as interesting biological phenomena such as gene duplications and lateral gene transfer. As we observed for the Apicomplexa and fungal data sets, our approach also serves as a means of identifying problematic gene trees which arise from horizontal gene transfer, paralogy, or experimental artifacts such as misannotations or misalignments. Furthermore, the scenario in our mixed coalescent distribution simulation—where the non-outlier trees are sampled from an unknown mixture of distributions—cannot be handled by parametric methods, with the possible exception of
the genome spectral methods. However, even the genome spectral methods ignore possible statistical dependencies between different feature spectra. In contrast, we propose analyzing a collection of gene trees without reducing gene trees to summarizing information. Our kdetrees approach also possesses a considerable advantage in speed over other methods, which is of paramount importance for a tool used in whole-genome phylogenetic analysis.

Simulations

The results of our simulations were generally positive for kdetrees. Although Phylo-MCOA was often able to slightly outperform kdetrees in classification accuracy, the difference was often relatively small. However, in terms of computational time, kdetrees vastly outperforms Phylo-MCOA, especially as the number of trees in the dataset increases.

In all cases studied, methods incorporating branch length information outperformed the topology only methods. The performance of the geodesic distance was better in the “single” simulations than the “mixed” simulations, although the reason for this is unclear. All of the methods were able to correctly identify the outlier tree when the effective population size (and thus tree variance) was low, provided that a suitable tuning parameter was chosen. As the variance of the coalescent trees increased, the performance of Phylo-MCOA tended to degrade at a slightly slower rate than kdetrees.

It should be noted that choosing a suitable tuning parameter can be quite difficult, as the optimal value depends on not only the details of the data set, but also one’s subjective opinions on the relative merits of the sensitivity and specificity of the classifier. As such, we also studied the behavior of the algorithms when using their default tuning parameters. This information is relevant, since many users will not change the parameters from their default values. With these values we found that kdetrees is slightly superior to
Phylo-MCOA in the single-distribution simulations. In the mixed-distribution simulations the default values for Phylo-MCOA resulted very poor performance, while kdetrees’s rate of outlier identification was much higher.

The third simulation set compared the distribution of scores for outlier trees to the scores of non-outlier trees. Although the distributions are not completely distinct, it is clear that the outlier trees tend to have scores smaller than the majority of non-outlier trees. Since the outlier trees were generated as completely random coalescent trees, there will inevitably be trees generated which have structure similar to the non-outlier trees, simply by chance, and this accounts for some of the overlap between the distributions. With real data, such trees would correspond to genes which have some exotic history, but nonetheless appear to have a phylogeny substantially similar to the rest of the genes in the genome. In this case, it is ambiguous whether or not such a gene should be legitimately classified as an outlier.

The main advantage of kdetrees over Phylo-MCOA lies in the vast improvement in running time on larger data sets. Although both appear to be polynomial time algorithms as implemented, the running time measurements suggest that our method implemented in the software kdetrees is a quadratic time algorithm, while the algorithm to identify outliers implemented in Phylo-MCOA is cubic time. For small data sets the difference is not significant, however for data sets with several thousand trees the Phylo-MCOA requires many hours to complete, while kdetrees will finish within a few minutes.

Fungal Data

The application of kdetrees to a set of 11 fungal protein alignments identified the contrived paralogous alignment of LolC and CysD and as an outlier. This was a case that
could easily arise in phylogenomic analysis, where OrthoMCL (Li et al. 2003) identified the genes as orthologs, though the group was subsequently broken into separate ortholog sets by application of COCO-CL (Jothi et al. 2006) to the OrthoMCL output. The CysD protein, encoded in genomes of many filamentous fungi, is believed to provide for scavenging toxic H2S, diverting it to cysteine and methionine biosynthesis pathways (Sieñko et al. 1998). It seems unlikely that obligately plant-symbiotic fungi, such as Epichloë, are likely to get significant exposure to H2S, so it is unsurprising that most have apparently lost functional copies. However, the closely related LolC protein is apparently an enzyme in the biosynthetic pathway to loline alkaloids, a group of insecticidal compounds important in host protection. Inspection of synteny relationships, and identification by BLAST of remnants of cysD that were not identified as genes by FGeneSH, indicated that LolC and CysD were indeed paralogous. Thus, the kdetrees result was expected and indicative of the utility of this program to identify outliers due to paralogy.

Apicomplexa Data

The phylum Apicomplexa contains many important protozoan pathogens (Levine 1988), including the mosquito-transmitted Plasmodium spp., the causative agents of malaria; T. gondii, which is one of the most prevalent zoonotic pathogens worldwide; and the water-born pathogen Cryptosporidium spp. Several members of the Apicomplexa also cause significant morbidity and mortality in both wildlife and domestic animals. These include Theileria spp. and Babesia spp., which are tick-borne haemoproteozoon ungulate pathogens, and several species of Eimeria, which are enteric parasites that are particularly detrimental to the poultry industry. Due to their medical and veterinary importance, whole genome sequencing projects have been completed for multiple prominent members of
the Apicomplexa.

We analyzed the data set presented in [Kuo et al. (2008)], which consists of 268 orthologous genes from seven species of Apicomplexa and one outgroup ciliate, *Tetrahymena thermophila*. To this set of genes we appended sequences from the Set8 gene. The Set8 gene has been identified by [Kishore et al. (2013)] as a probably case of horizontal gene transfer from a higher eukaryote to an ancestor of the Apicomplexa. Supplementary Figure S.3 illustrates the scores that the method generated for the trees in the data set.

Our analysis of the data set presented in [Kuo et al. (2008)] identified several putative outlier trees. Supplementary Figure S.3 illustrates the scores that the method generated for the trees in the data set. Of the trees identified by the dissimilarity map method, it seems that most are likely attributable to either incorrect annotation or the inclusion of non-orthologous genes. The most common culprits were sequences from *Eimeria tenella* (Et). (See Table 1 and Supplementary Table S.1 for more details.) The results from the topological dissimilarity method were less decisive. Here there were no clearly identifiable problems with the trees or sequences in most cases identified as putative outliers. This result is similar to that found in the simulation studies, suggesting that the incorporation of the branch length information by the dissimilarity map provides superior results.

While the Set8 gene was not identified initially by kdetrees as an outlier gene, its score was very close to the classification threshold, and is the next gene to be classified as an outlier if the tuning parameter is lowered slightly, from 1.5 to 1.3. Since many of the outliers in the analysis seem to be caused by questionable annotation in the Et sequences, we removed these sequences from the data set and generated new gene trees. In the new analysis, the Set8 gene was identified as the furthest outlier tree.

These results demonstrate the potential applicability of the kdetrees method to
the curation of genetic data sets, by providing a simple tool for highlighting sequences or alignments that may be of further interest. The successful identification of the Set8 outlier, suggests that our method is able to draw attention to cases of interesting biological phenomena.

Future work.—With the advent of ever-cheaper means for whole genome sequencing, there is a plethora of data that is available for phylogenomic analysis. One of the great opportunities offered by modern genomics is that phylogenetics applied on a genomic scale (phylogenomics) should be especially powerful for elucidating gene and genome evolution, relationships among species and populations, and processes of speciation and molecular evolution. However, a well-recognized hurdle is the sheer volume of genomic data that can now be generated relatively cheaply and quickly, but for which analytical tools are lagging. There is a major need to explore new approaches to undertake comparative genomic and phylogenomic studies much more rapidly and robustly than existing tools allow.

We are interested in developing a phylogenomic pipeline that is convenient and accessible, as well as robust. To accomplish this aim, important problems that need attention are (1) refinement of gene calls based on comparison among orthologs from multiple genomes, and (2) comparing thousands of gene phylogenies across whole genomes. In order to achieve these goals we have to have phylogenetic analysis tools very efficient in computational time as well as memory. Therefore, in the development of our software kdetrees, we focused on the efficiency of the algorithm in terms of computational time as well as memory space to make it even more attractive for comparative genomics and to incorporate the method into a pipeline for genome-wide phylogenetics as an annotation supplement and to discover evolutionary processes that deviate significantly from tracking species trees.
In future work we intend to extend our method to clustering trees based on similarity, in addition to identifying outliers. The identification and exclusion of outlier points is an important preliminary step in many clustering methods. The removal of outlier points facilitates better inference in at the clustering stage (Camastra and Verri 2005; Hur et al. 2000, 2001).

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## Table 1. Apicomplexa gene sets identified as outliers by *kdetrees*.

| No. | GeneID 2 | Functional Annotation                                      |
|-----|----------|-----------------------------------------------------------|
| 488 | PF08_0086| RNA-binding protein, putative                            |
| 497 | PF13_0228| 40S ribosomal subunit protein S6, putative               |
| 515 | PFA0390w | DNA repair exonuclease, putative                          |
| 546 | PFF0285c | DNA repair protein RAD50, putative                        |
| 547 | PFL1345c | Radical SAM protein, putative                             |
| 641 | PFE0750c | hypothetical protein, conserved                           |
| 660 | PF10_0043| ribosomal protein L13, putative                           |
| 662 | PF11_0463| coat protein, gamma subunit, putative                     |
| 728 | MAL13P1.22| DNA ligase 1                                              |
| 747 | PFB0550w | Peptide chain release factor subunit 1, putative          |
| 773 | PFF0120w | putative geranylgeranyltransferase                        |
| 780 | PFD0420c | flap exonuclease, putative                                |

1Based on geneset designations in [Kuo et al. (2008)](#).
2Geneset represented by GeneID for *Plasmodium falciparum*. 
Figure Captions

Figure 1. The species trees used to generate gene trees under the coalescent model for the simulation experiments. At top-left is the tree used for the “single” coalescent distribution simulations, while the other trees are used in the “mixed” simulations.

Figure 2. Summary of the design of the simulation comparing kdetrees and Phylo-MCOA. (See Figure 1 for a plot of the species tree used.) The non-outlier trees were generated under two models, the “single” coalescent model, and the “mixed” model. This process was replicated many times, and the count of successful identifications for each method recorded.

Figure 3. Summary of the simulation design for the simulation comparing the tree score distributions for outlier trees and non-outlier trees.

Figure 4. ROC curves comparing kdetrees and Phylo-MCOA as the classification tuning parameter is varied. The effective population size is 2000 for the coalescent trees. At left are the “single” contained coalescent simulations, with the non-outlier trees all contained within a single species tree. At right are results from a “mixed” simulation, with the non-outlier trees generated from a mixture of 5 species trees.

Figure 5. Summary of simulation results comparing performance of kdetrees and Phylo-MCOA for various values of the effective population size. Shown is the proportion of simulated data sets in which the methods identified the outlier tree. Top two plots use default tuning parameters, while the bottom two plots use tuning parameters chosen based on results of the ROC simulation. For kdetrees the tuning parameter was $\kappa = 0.7$, and for Phylo-MCOA it was $\kappa = 0.25$. 
Figure 6. Kernel density estimates of the observed distribution of tree scores. The “coalescent” scores are for contained coalescent trees generated within a fixed species tree (bottom). A single random outlier tree is added to this data set and its score computed. This process is replicated to generate the sample of “outlier” tree scores (top). Lines and dots represent the 5%-95% quantiles and the median, respectively. An effective population of 2000 was used to produce these estimates.

Figure 7. The running time (in hours) of kdetrees and Phylo-MCOA as the number of trees in the data set increases. The trees used here have 50 tips each.
FIGURES
20 coalescent trees contained in species tree 1

20 coalescent trees contained in single species tree 3

20 coalescent trees contained in single species tree 5

20 coalescent trees contained in single species tree 2

20 coalescent trees contained in single species tree 4

100 coalescent trees contained within Single species tree

Non-outlier trees

1 random outlier tree

1 sample (N=101)

kdetrees

Phylo-MCOA

Count true and false identifications
500 coalescent trees contained in one species tree

Evaluate density at each observed tree and estimate non-outlier score distribution

```
kdeTrees
```

Estimate of density function

Evaluate densities of outlier trees and Estimate outlier score distribution

500 random coalescent trees
method

|          | single       | mixed        |
|----------|--------------|--------------|
|          | FPR          | TPR          |
|          |              |              |
|          |              |              |
|          |              |              |
|          |              |              |
| kdetrees (geodesic) | |              |
| kdetrees (dissimilarity) | |              |
| pMCOA (dissimilarity) | |              |
Nonparametric Estimation of Phylogenetic Distributions

PROPORTION OF OUTLIERS IDENTIFIED

Dist: geodesic, topological, dissimilarity
Method: kdetrees, pMCOA

Proportion of Outliers Identified

$\text{Proportion of Outliers Identified}$

$\text{n}_{\text{eff}}$

$\text{Proportion of Outliers Identified}$

$\text{n}_{\text{eff}}$

$\text{Proportion of Outliers Identified}$

$\text{n}_{\text{eff}}$

$\text{Proportion of Outliers Identified}$

$\text{n}_{\text{eff}}$
Supplemental Material
## Table S.1. Analysis of Apicomplexa gene-sets identified as outliers

| Gene ID   | Functional Annotation          | Analysis                                                                                                                                 |
|-----------|--------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| PF08_0086 | RNA-binding protein, putative  | Significant sequence length disparity (164 a.a. for Ta vs 1075 a.a. for Pf). Generally good sequence alignment in one region of 100 residues; otherwise, alignment is poor. |
| PF13_0228 | 40S ribosomal subunit protein S6, putative | Tt sequence much longer than all others; long N-terminal and C-terminal extensions. Very good alignment in blocks, but with lengthy insertions for outgroup Tt. Possible incorrect annotation of Tg sequence. |
| PFA0390w | DNA repair exonuclease, putative | Short sequences for Et and Cp. Several homopolymer stretches in Et. Modest to good alignment in multiple blocks, Et being an exception in several regions. Possible incorrect annotation of Et sequence. |
| PFF0285c | DNA repair protein RAD50, putative | Poor alignment in general. Three modest blocks (50-100 aa) of reasonable sequence alignment. Et sequence contains long homopolymeric stretches. Pf and Pv have long insertions that might be translated introns. |
| PFL1345c | Radical SAM protein, putative  | Relatively short sequence for Et. Homopolymeric stretch at N-terminus of Tg. Modest to good alignment in blocks.                            |
| PFE0750c | hypothetical protein, conserved | Large difference in sequence lengths; 269 residues for Et vs. 848 for Pf. Central region with modest to good alignment; Et exhibited poor sequence identity suggesting it might not be a homologue. |
| PF10_0043 | ribosomal protein L13, putative | 80 residue N-terminal extension in Tg. Good sequence alignment, with Tt (outgroup) being an exception. Tt sequence might not be a homologue. |
| PF11_0463 | coat protein, gamma subunit, putative | Multiple homopolymer stretches in Et sequence. Generally good alignment for all but Et; sequence might not be homologous. |
| MAL13P1.22 | DNA ligase 1                      | Homopolymer stretches in Et sequence with poor alignment to other sequences. Et sequence might be incorrectly annotated and/or might not be homologous. |
| PFB0550w | Peptide chain release factor subunit 1, putative | Short sequence for Et (132 residues), with long homopolymer stretch. Other sequences are approximately 425 a.a. in length. Generally good alignment, even for Et over a short region (50 residues). Possible incorrect annotation of Et sequence. |
| PFF0120w | putative geranylgeranyltransferase | Two homopolymer stretches (serine) in Et sequence. Moderately good alignment. Possible incorrect annotation of Et sequence. |
| PFD0420c | flap exonuclease, putative      | Very discrepant sequence lengths; 179 a.a. for Et vs. 2213 a.a. for Tt. All other sequences 500 - 600 residues in length. Good alignment over several regions, although sequence for Et is absent in portions of these regions. Very long N-terminal extensions and insertions in Tt sequence. Possible incorrect annotations for Et and Tt. |

Pf = *Plasmodium falciparum*, Pv = *Plasmodium vivax*, Bb = *Babesia bovis*, Ta = *Theileria annulata*, Et = *Eimeria tenella*, Tg = *Toxoplasma gondii*, Cp = *Cryptosporidium parvum*, and Tt = *Tetrahymena thermophila* (outgroup).
Figure S.1. Schematic of how trees are converted to vectors. Numbers on branches in the unrooted tree are branch lengths. In this example, the tree is first converted to either a branch length-based dissimilarity map (matrix of distances between tips) or topological dissimilarity maps (matrix of number of edges between tips). Moving from left to right across rows in one half of a matrix, values are placed into a single column to yield a vector of distances between tips in the tree.
Figure S.2. Plots of the first 4 Apicomplexa gene trees identified as outliers. The extremely long branches lead to the identification as outliers, and are likely the result of incorrect annotations of the original sequences.
**Figure S.3.** Summary of tree scores for the Apicomplexa data set. In the top row the scores of individual trees are shown. “Tree index” refers to the ordering of the trees in the input files. In the bottom row, the scores are summarized as a histogram. In the left column are the results computed with branch-length information, while the topology-only results are shown at right.