

Running Head: NEW ALGORITHMS AND METHODS IN PHYML 3.0

New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0

Stéphane Guindon^{1,2}, Jean-François Dufayard¹, Vincent Lefort¹, Maria
Anisimova^{1,3,4}, Wim Hordijk^{1,5}, and Olivier Gascuel^{*,1}

- 1: *Méthodes et Algorithmes pour la Bioinformatique, LIRMM, CNRS – Université de Montpellier, France*
- 2: *Department of Statistics, University of Auckland, New Zealand*
- 3: *Institute of Computational Science, ETH Zurich, Switzerland*
- 4: *Swiss Institute of Bioinformatics, Lausanne, Switzerland*
- 5: *Department of Statistics, University of Oxford, UK*

**Corresponding author:*

Olivier Gascuel, Méthodes et Algorithmes pour la Bioinformatique, LIRMM, CNRS – Université de Montpellier, 161 rue Ada, 34392 Montpellier Cedex 5 – France.

gascuel@lirmm.fr, <http://www.lirmm.fr/~gascuel>

ABSTRACT

PhyML is a phylogeny software based on the maximum-likelihood principle. Early PhyML versions used a fast algorithm performing Nearest Neighbor Interchanges (NNIs) to improve a reasonable starting tree topology. Since the original publication (Guindon and Gascuel, 2003), PhyML has been widely used (>2,300 citations in ISI Web of Science), because of its simplicity and a fair compromise between accuracy and speed. In the meantime research around PhyML has continued, and this article describes the new algorithms and methods implemented in the program. First, we introduce a new algorithm to search the tree space with user-defined intensity, using Subtree Pruning and Regrafting (SPR) topological moves. The parsimony criterion is used here to filter out the least promising topology modifications with respect to the likelihood function. The analysis of a large collection of real nucleotide and amino-acid data sets of various sizes demonstrates the good performance of this method. Second, we describe a new test to assess the support of the data for internal branches of a phylogeny. This approach extends the recently proposed approximate likelihood-ratio test (aLRT) and relies on a non-parametric, Shimodaira-Hasegawa-like procedure. A detailed analysis of real alignments sheds light on the links between this new approach and the more classical non-parametric bootstrap method. Overall, our tests show that the last version (3.0) of PhyML is fast, accurate, stable and ready to use. A web server and binary files are available from <http://www.atgc-montpellier.fr/phyml/>

Keywords: phylogenetic software, maximum likelihood, tree search algorithms, NNI, SPR, branch testing, LRT and aLRT, bootstrap analysis.

Supplementary Material: http://www.lirmm.fr/mab/phyml_benchmarks/

Likelihood-based methods of phylogenetic inference, including maximum-likelihood and Bayesian approaches, have been shown to be accurate in a number of simulation studies (e.g. Kuhner and Felsenstein, 1994; Ranwez and Gascuel, 2001; Guindon and Gascuel, 2003). They are now commonly considered as the best approach, compared to other methods (e.g. distance or parsimony). However, maximum-likelihood (ML) approaches involve complex calculations and tend to be slow. Until the end of the 90's, they were generally limited to the analysis of small data sets, typically with a few dozens of taxa, a single gene, and no bootstrap. Since that time, ML algorithms have improved considerably (and computers are much faster!). Our original PhyML algorithm (Guindon and Gascuel, 2003) performs simultaneous Nearest Neighbor Interchanges (NNIs) to improve a reasonable starting tree (typically inferred with a fast distance or parsimony method). Its speed enables heavy bootstrap studies and the analysis of large data sets, with hundreds of taxa and thousands of sites from concatenated genes. PhyML is widely used (>2,300 citations in ISI Web of Science), due to its fair accuracy, speed and simplicity of use. Besides PhyML, other fast ML programs were developed. METAPIGA (Lemmon and Milinkovitch, 2002), TREEFINDER (Jobb et al., 2004) and GARLI (Zwickl, 2006) use genetic algorithm-based approaches. IQPNNI (Vinh and von Haeseler, 2004) extends the PhyML algorithm by removing and repositioning taxa. RAxML (Stamatakis, 2006a) implements highly-optimized heuristics to search the tree space using Subtree Pruning and Regrafting (SPR) topological moves. LEAPHY (Whelan, 2007) provides a number of non-deterministic heuristics to intensify the tree search. FastTree 2.1 (Price et al., 2009) is a fast approximate approach for estimating ML trees. Last but not least, MrBayes (Ronquist and Huelsenbeck, 2003) MCMC Bayesian program is fairly fast with standard data sets. A number of other likelihood-based programs can be found on Joe Felsenstein's web page¹.

Since its original 2003 publication, research around PhyML has continued. Firstly, some of our users pointed out that with large and difficult data sets (typically obtained from concatenated genes) NNI tree searches sometimes get trapped in suboptimal local maxima of the likelihood function. When this occurs, the resulting trees may still show some of the defects of the starting trees (e.g. long-branch attraction). We designed PhyML-SPR (Hordijk and Gascuel, 2005) to address this issue. PhyML-SPR relies on SPR moves to explore the space of tree topologies. Because such an approach is highly time consuming, we implemented several heuristics to avoid useless computations.

¹ <http://evolution.genetics.washington.edu/phylip/software.html>

The main heuristic uses a fast distance-based approach that detects the least promising candidate SPR moves, which are then simply discarded. Moreover, the change in likelihood for any remaining potential SPRs is locally estimated, as opposed to globally evaluating the entire tree for each candidate move. These heuristics are combined to efficiently select potential SPRs and to concentrate most of the likelihood computation on the most promising moves.

Secondly, although bootstrap proportions can be estimated with fast ML programs, this task is still highly time demanding and not necessarily relevant in certain contexts, for example in exploratory stages. We thus revisited the null branch tests (for a review see Felsenstein, 2003, pp. 319-320). The approach proposed by Anisimova and Gascuel (2006) compares the current subtree configuration around the (internal) branch of interest to the two alternatives defined by NNI moves around this branch. It computes the likelihood of these two alternatives locally, and performs an approximate likelihood-ratio test (aLRT) based on the log-ratio between the likelihood value of the current tree and that of the best alternative. The corresponding software (PhyML-aLRT) is fast, as computing the aLRT support of all tree branches does not significantly increase the computing time required to build the tree.

These two studies were developed independently and gave rise to different versions of PhyML. In an effort to integrate these novelties in a ‘unified’ version of the program, new research led us to revise and enhance the original SPR and aLRT approaches. The purpose of this article is to describe these new methods and evaluate their performance with simulated data and a dedicated benchmark of 120 real-world nucleotide and amino-acid alignments extracted from TreeBase (Sanderson et al., 1994). The first part of the paper introduces a new SPR-based tree search algorithm and examines its performance compared to other popular algorithms. The second part focuses on the aLRT statistic, its parametric and non-parametric interpretations, and compare the results to those of the standard bootstrap.

PART I: STRATEGIES TO SEARCH THE TREE SPACE

Methods and Algorithms

New SPR algorithm. PhyML originally relied on NNI moves to explore the space of tree topologies, starting from a fast distance-based tree (see Guindon and Gascuel, 2003). Simulation results showed that the phylogenies estimated using this approach are generally as likely as those

returned by the then popular (but slow) approach implemented in the software fastDNaml (Olsen et al., 1994). However, maximizing the likelihood function may be much more challenging when analyzing real data sets. Indeed, the landscapes defined by the likelihood function with real data sometimes seem to be very rugged, especially when the phylogenetic signal is poor and/or conflicting across data partitions. A standard solution to overcome this problem is to focus on SPR moves instead of NNIs. The neighborhood of trees defined by possible SPR moves to any given topology is much wider than the neighborhood defined by NNI moves. SPRs are thus more efficient than NNIs in jumping across very distinct tree topologies in just one step, therefore increasing the ability to avoid local maxima of the function to be optimized. However, better performance in terms of tree topology exploration comes at the price of a heavier computational burden. With standard greedy algorithms, most SPR moves correspond to very unlikely trees. An efficient algorithm should therefore rely on an accurate filter to dismiss such useless SPR moves before evaluating the likelihood.

PhyML-SPR (Hordijk and Gascuel, 2005) implements a fast distance-based approach to filter out the least promising SPR moves. The analysis of real-world data sets with a crude implementation of this approach showed that it performs well compared to RAxML. However, other filters, not necessarily relying on distances between sequences, could be applied here. Parsimony-based criteria are particularly appealing because parsimony and likelihood are strongly interconnected from a theoretical perspective (minimizing the parsimony score is equivalent to maximizing the likelihood under some assumptions, see Theorem 2 in Penny and Steel, 2000). Hence, the least promising SPR moves with respect to the likelihood function can be detected through the corresponding changes in parsimony scores.

PhyML 3.0 implements a new SPR search algorithm that relies on a parsimony-based filter instead of a distance-based one. The algorithm `One_Spr_Cycle` is summarized in Appendix A. It processes every subtree in the current phylogeny. For each subtree, the parsimony score at each regraft position is evaluated. The corresponding parsimony scores are then sorted (steps A-D). The likelihoods for the most parsimonious solutions are evaluated next using a two-level approach. At the first level (step G4), the likelihood of the tree is evaluated after applying a parsimonious SPR move without adjusting any branch length. If this likelihood is greater than the likelihood for the best move found so far, the current move becomes the best move (step G5). Otherwise, the three branch lengths at the regraft position are optimized so as to maximize the likelihood (step G6). Note that these approximate estimations rely on updating the likelihood of a limited number of subtrees rather than

updating the whole data structure. Hence, these steps are relatively fast. Once all SPR moves have been evaluated, if the likelihood for the best SPR move is greater than the likelihood of the current phylogeny, this move is applied and the data structure is completely updated (steps H-I).

Only one tuning parameter in `One_Spr_Cycle` cannot be estimated from the data and needs to be fixed a priori. This parameter, PT, corresponds to the maximum number of parsimony steps which can be added by the SPR under scrutiny to the current tree parsimony; for example, $PT = 0$ implies that applying a SPR move must not increase the parsimony score of the current tree, otherwise its likelihood score is not calculated. The value of PT determines the number of SPR moves for which likelihood scores are actually calculated. The lower PT, the smaller the number of SPR moves to be considered for likelihood calculations. On the opposite, with large PT values, the parsimony filter has not effect and the likelihood of all SPRs are calculated in subsequent steps. Several real-world data sets were analyzed with various values for this parameter in order to identify the best trade-off between likelihood maximization and computation time (see the section 'Accuracy of the parsimony filter').

The algorithm `One_Spr_Cycle` takes as input a phylogenetic tree and outputs a modified phylogeny with likelihood greater than or equal to the likelihood of the input tree. Depending on the data, it performs one, several, or no SPR moves. Branch lengths and tree topology are the only parameters that can be modified at this stage. `One_Spr_Cycle` is the core of a higher-level algorithm `Multiple_Spr_Cycles` sketched in Appendix B. This algorithm first processes every subtree through `One_Spr_Cycle` (step B). It then adjusts the parameters of the substitution model such as the shape of the gamma distribution of rates across sites or the relative rates of the GTR model (step C). Next, all branch lengths are adjusted using a simple optimization method based on Brent algorithm (step D), just as in the original PhyML algorithm. The whole data structure is updated after that (step E). These three steps are iterated until no further improvement of the likelihood is found. For DNA data sets, additional rounds of `Multiple_Spr_Cycles` without filtering the SPR moves using parsimony are applied. While this additional step involves the calculation of likelihoods for large sets of SPR moves and therefore slows down the tree inference overall, it provides significant improvements in terms of likelihood maximization (with DNA only; this procedure is not applied to proteins, as we did not observe any clear advantage). Once no improvement of the likelihood is found using `Multiple_Spr_Cycles`, simultaneous NNIs are performed, just as in the original PhyML algorithm. The search is completed using refined NNI steps with full optimization of the five branch

lengths being involved in the move, instead of optimizing the central branch length only as in (Guindon and Gascuel, 2003). This last procedure not only improves tree searching compared to simultaneous NNIs, but also forms the basis of aLRT branch support calculations, as we shall further explain. The search is stopped when no notable likelihood improvement is found between two full NNI steps.

The main difference between this SPR-based algorithm and the one previously proposed by Hordijk and Gascuel (2005) is the use of a parsimony rather than a distance-based SPR filter. We shall see that this change yields a sensible improvement in likelihood optimization and run time. This result is not surprising because parsimony is usually more correlated to likelihood than tree length is (results not shown). Therefore, finding ‘good’ SPR moves is made easier and the computational burden is reduced. Moreover, the filter relies on Fitch parsimony, the implementation of which can easily be optimized for speed. Indeed, the core of the parsimony score evaluation relies on comparing bit vectors of length four (for nucleotides) or twenty (for amino acids), which can be done very efficiently with modern computers. Note however that Fitch parsimony uses uniform weights for different substitution events. This limitation could be problematic with proteins for which substitutions are much more frequent for specific pairs of amino acids compared to others (e.g. Le and Gascuel, 2008). It is also worth mentioning that the core of the SPR algorithm implemented in PhyML (Appendix A) does not involve adjusting all branch lengths in the tree. Only local and approximate adjustments are applied, which saves a considerable amount of computing time. A similar “lazy” approach is used in RAxML.

Our SPR algorithm comes in different flavors: (1) the ‘SPR’ option relies on the algorithm described above, starting from a BioNJ (Gascuel, 1997) or a minimum parsimony (MP) tree; (2) ‘BEST’ runs both the SPR and NNI algorithms and outputs the best of the two resulting trees (usually the SPR tree, but not always, see below); (3) ‘RAND’: trees inferred by ‘SPR’ starting from random trees (instead of BioNJ or MP) can also be added to the ‘BEST’ option. PhyML then outputs the best of all inferred trees (these are written to a file so one can check the likelihood landscape, depending on whether the same tree is found in all runs, or a number of different topologies are found).

Phylogenetic methods comparison criteria. Defining relevant criteria to get a fair comparison between phylogenetic methods is a difficult task. In this study, we are mostly interested in comparing the ability of different reconstruction methods to maximize the likelihood function, and how long it

takes to perform this task. Comparing computation times is relatively straightforward provided that all tests are conducted on the same computer in the same conditions. Comparing likelihoods proved to be more complex. For each data set, the likelihoods obtained by the different methods were sorted and the rank of each method was deduced. These ranks were then corrected in order to eliminate numerical effects, i.e. the same topologies are given the same rank, even if their likelihood values (slightly) differ. Indeed, our work on SPR-based algorithms mostly focuses on the ability of these approaches to find the ‘best’ tree topologies, and small differences in likelihoods not related to differences in topologies are of no interest in this context. The likelihoods of two phylogenies that share the same topology should therefore be rated equally. Another variable of interest is the number of times a method fails to find a phylogeny which log-likelihood is close to the highest log-likelihood found by any of the methods being compared. We thus counted the number of data sets for which the log-likelihoods returned by a given method was smaller than the highest log-likelihood found on the corresponding alignments minus 5.0. While this boundary of 5.0 points of log-likelihood is arbitrary, we believe that it provides a simple and practical way to tell the methods apart at first sight. To complete this first rough assessment, we also used a Shimodaira-Hasegawa (1999) test to check whether the log-likelihood obtained with each method and each data set was significantly smaller than the log-likelihood of the most likely tree found on the corresponding alignments. Average topological distances between each tree and the corresponding most likely trees were also evaluated in order to assess the impact of differences of log-likelihood in terms of reconstructed topologies. With simulated data, we compared the true topology to the inferred one.

Data

Real data. Our comparison of phylogenetic methods mostly relies on a dedicated benchmark of real data sets extracted from Treebase (Sanderson et al., 1994). This benchmark contains two groups of alignments that differ in size. Each DNA alignment in the first group (medium-size data sets) comprises between 50 and 200 nucleotide sequences shorter than 2,000 sites. Each protein alignment has between 5 and 200 sequences, shorter than 2,000 sites. Based on these size criteria, we selected the 50 most recent DNA alignments and the 50 most recent protein alignments registered in Treebase. The second group of data sets is made of larger alignments. Each alignment has at least 300 and 40 sequences per DNA and protein alignments respectively and no limit of length is imposed. The most

recent alignments registered in Treebase were considered here again, resulting in 10 DNA and 10 protein alignments.

Simulated data. We also analyzed simulated data similar to those used in the original PhyML publication (Guindon and Gascuel, 2003). The randomly generated 40-taxon trees were identical to the original ones, while the sequences evolving along these trees were simulated using a different substitution model. We used a GTR model with four gamma-distributed rate categories (see Supplementary Material for simulation details), instead of the original Kimura two-parameter model without rate across sites variability. This model is the only one that PhyML and RAxML, the two programs compared in this study, have in common. Simulated data do not provide the most relevant way to compare the ability of different methods to maximize the likelihood function, as the likelihood landscape tends to be smoother than with real data. However, knowing the true phylogenetic model, that is, the model that actually generated the sequences, allows for comparison of methods based on their ability to recover the truth, which is of great interest. In particular, it becomes possible to compare the estimated and true tree topologies.

All these real and simulated data sets along with additional information (e.g. simulation parameters, true trees used in simulations, etc.) are available from the Supplementary Material web page http://www.lirmm.fr/mab/phyml_benchmarks/.

Results

Accuracy of the parsimony filter. The performance of the new SPR algorithm proposed here relies on the accuracy with which the parsimony filter ranks SPR moves with respect to the likelihood function. A first important question was to determine whether this filter performs better than the distance-based criterion implemented in PhyML-SPR (Hordijk and Gascuel, 2005). We therefore compared the latest version (3.0) of PhyML to PhyML-SPR using 50 DNA and 50 protein medium-size data sets (see above). We also compared different stringencies of the parsimony filter by varying the value of the parameter PT, which governs the number of SPR moves that are evaluated using the likelihood criterion. A value of 0 for PT indicates that only SPR moves at least as good as the current tree with respect to parsimony will be subsequently evaluated. $PT = \infty$ means that the likelihoods of all the SPR moves will be evaluated.

The results presented in Table 1 show that the parsimony criterion outperforms the distance-based one overall. Indeed, the average log-likelihood ranks for the parsimony-based approaches are

notably lower than the ranks obtained for the distance-based approach. Also, for DNA data sets, the distance-based trees are at least 5 log-likelihood points below the most likely solutions (among the 4 estimated trees per alignment) for 15 data sets out of 50, i.e. 30%. This difference is similar but less pronounced on protein alignments. The reason why the two criteria do not perform the same on DNA and proteins is likely related to the fact that parsimony is well suited (and widely used) with nucleotide sequences, but questionable with protein alignments due to the difficulty to define substitution costs with amino acids (e.g. Felsenstein, 2003, pp. 97-100). Moreover, the SH test is rarely significant (3 times among 100 datasets), indicating that the gain obtained by the new SPR algorithm is appreciable but often insufficient to produce statistically better topologies. Finally, the topological distance between the trees of distance-based PhyML-SPR and those of the new parsimony-based version is relatively high (~ 0.2), both with DNA and protein alignments, meaning that the likelihood improvement that we obtain with the new version does change the topology of inferred trees.

Table 1 also shows that phylogenies estimated with $PT=5$ (i.e., SPR moves with corresponding parsimony score no more than 5 steps above the parsimony of the current tree) are generally more likely than those estimated by setting $PT=0$ (i.e., any SPR move augmenting the parsimony value is discarded). Also, the log-likelihood obtained with $PT=5$ and $PT=\infty$ (i.e., there is no parsimony filter: the likelihood of every SPR move is evaluated) are similar, which suggests that $PT=5$ is a sensible value for this parameter. Comparisons of computing times (see Supplementary Material) indicates that $PT=0$ and $PT=5$ have similar speed, while $PT=\infty$ is significantly slower. Altogether, these observations plus additional tests of intermediate values for PT (results not shown) indicate that $PT=5$ is a good trade-off between likelihood maximization and computational burden. $PT=5$ is thus used in all further experiments and is the default value in PhyML 3.0.

Analysis of simulated data sets. We analyzed 100 simulated data sets similar to the ones that were used in the original PhyML publication. The performance of the new NNI-based algorithm (simultaneous NNIs are completed by NNIs with 5-branch-length optimization, see above) and of the SPR-based options ('SPR', 'BEST', and 'RAND' with 5 random starting trees) were compared to the original (simultaneous NNIs only) PhyML algorithm (version 2.4.5) and RAxML (version 7.0). RAxML was used with the 'GTRGAMMA' option that runs a GTR model (Lanave et al., 1984) with 4 gamma distributed rate categories (Yang, 1993). The same model (GTR+ Γ 4) was used with all PhyML versions and options. The results are displayed in Table 2.

We notice a gap between the original NNI algorithm and the latest one with respect to average log-likelihood ranks (column 'Av. LogLk rank'). Indeed, the new approach estimates phylogenies that are generally more likely than those returned by the original approach. This observation confirms the benefit of adding fully optimized NNIs steps to the simultaneous NNIs procedure. For the same criterion, the new NNI algorithm performs even slightly better than both SPR-based algorithms 'SPR' and RAxML (3.59 vs. 3.71 and 3.86 respectively). This finding confirms our results in (Guindon and Gascuel, 2003), where we did not observe significant differences between NNI- and SPR-based tree search methods with simulated data. We shall see that with real data the picture is quite different. Moreover, regarding the 'Delta>5' criterion, SPR-based algorithms tend to be better than NNI-based ones which in a few cases (3-4/100) find topologies with log-likelihood smaller by 5 points than the best solution. RAND (taking the best of 'NNI', 'SPR' and 5 SPR-based searches with random starting trees) is clearly the most accurate approach as it outputs the most likely tree with most alignments (97/100). However, none of the observed differences in likelihoods is statistically significant using a SH test (column ' p -value<0.05'), which is not a surprise as these differences are usually very small (column 'Delta>5'). Hence, it is not a surprise that the average distance to the true tree topology is virtually the same for all methods (i.e., ~90% of the internal edges are correctly inferred on average, column 'Av. RF distance'), suggesting that the search for even more likely phylogenies may not be relevant for this type of data. However, as noted above, these data sets are probably 'too easy' and a fair comparison of phylogenetic reconstruction methods should involve the analysis of real-world data sets.

Ability to maximize the likelihood on real-world data sets. For nucleotide sequence alignments, we used a GTR+ Γ 4 model for all the compared methods and options, that is, the original NNI-based PhyML (2.4.5), the new 'NNI', 'SPR', 'BEST' and 'RAND' (with 5 random starting trees) options implemented in PhyML 3.0, and RAxML. The latter was run with 'GTRGAMMA' option, which corresponds to GTR+ Γ 4 as used with PhyML and standard in most phylogeny packages. In order to make sure that the likelihood scores returned by RAxML and PhyML are comparable, we used PhyML to evaluate the likelihoods of the phylogenies built with RAxML after re-estimating the numerical parameters of the model (i.e., branch lengths, gamma shape parameter and GTR relative rates). For protein data sets, the model used was WAG (Whelan and Goldman, 2001) combined with a discrete gamma distribution with four categories. Here again, only the tree topologies estimated with RAxML were conserved and the numerical parameters were re-estimated with PhyML in order to make log-likelihoods comparable. This re-estimation of numerical parameters is important as all programs (slightly) differ in the way they optimize and compute the tree likelihood. Tables 3 summarizes the performance of the different approaches regarding likelihood maximization on

medium-size data sets. Table 4 displays the results obtained on large data sets. Moreover, in Supplementary Material we provide a comparison of PhyML (+ Γ 4) with RAxML (option 'GTRMIX') and FastTree 2.1 (Price et al., 2009), both running the CAT approximation of the gamma rate distribution with four bins (Stamatakis, 2006). This approximation is much faster (~4 times) than the standard mixture model approach, but resulting topologies are expected to have lower likelihoods.

The results displayed in Table 3 show that the new version of 'NNI' slightly but consistently outperforms the original one for medium-size data sets. Indeed, the average log-likelihood ranks and the Robinson and Foulds distances are in favor of the latest version of 'NNI'. We also see that SPR-based methods outperform the NNI-based heuristics; for example, NNI-based trees have log-likelihood values smaller by >5 points than the best SPR-based solutions with 33/50 DNA and 20/50 protein alignments (see 'Delta>5' column). For the same reason, the 'SPR' option and the best of 'SPR' and 'NNI' perform similarly, even though 'NNI' outperforms 'SPR' in a few but noticeable cases, justifying the 'BEST' option.

When considering DNA data sets, the results of 'SPR', 'BEST' and RAxML are similar, with a slight advantage to 'SPR' and 'BEST' which tend to outperform RAxML regarding the average log-likelihood ranks (~2.8 and ~2.7 for 'SPR' and 'BEST', respectively, versus 3.2 for RAxML). However, all these methods almost always estimate trees with log-likelihoods very similar to the highest observed log-likelihoods ('Delta>5' column). The SH tests and comparison of tree topologies (' p -value<0.05' and 'Av. RF distance' columns) also confirm the virtually identical performance of these three approaches. Even NNI-based trees have log-likelihood values that do not significantly differ from the best ones in most cases (~90%; ' p -value<0.05' column). This likely explains the popularity of this approach, which is very fast and thus provides a relevant speed/accuracy compromise. Only 'RAND' seems to outperform all the other methods in terms of likelihood maximization. This approach returns the most likely tree among the 6 tested methods with most alignments (47/50).

The picture is roughly the same with protein data sets. 'SPR', 'BEST' and RAxML display similar performance with a slight advantage to RAxML when considering the average log-likelihood ranks, Delta>5 and RF criteria. Here again, 'RAND' outperforms the other approaches and generally returns the most likely tree (45 alignments among 50). Moreover, all compared methods, including NNI-based ones, find trees that do not significantly differ from the best tree using a SH test (' p -value<0.05' column).

For large data sets, Table 4 shows that with DNA alignments SPR-based methods perform significantly better than NNI-based ones. Indeed, the log-likelihoods returned by SPR-based methods are systematically at least 5 points greater than the log-likelihoods estimated by the two NNI-based methods, and the difference is statistically significant in most cases. ‘SPR’ and RAxML perform approximately the same with a slight advantage for ‘SPR’ here again. Due to limitations in terms of the computing time available, we did not evaluate the performance of ‘BEST’ and ‘RAND’. For example, our largest DNA data set (1,556 taxa and 915 sites) required approximately 80 hours of computation with ‘SPR’ (see Supplementary Material for details). For large protein data sets, the contrast between methods is less pronounced. The new PhyML 3.0 version of NNI outperforms the original one (regarding both likelihood ranks and topological distances) and is similar to ‘SPR’, while RAxML performs very well as it systematically returned the most likely tree. However, all these differences are rarely (3 times among 10 alignments) significant when comparing NNI-based and SPR-based approaches, and never significant with ‘SPR’ versus RAxML.

The log-likelihood differences are larger (column ‘Delta>5’) and more often significant (column ‘ p -value<0.05’) with these large data sets compared with medium-size data sets (Table 2). Both findings are expected, as larger numbers of taxa and sites implies larger absolute log-likelihood values. Also, Robinson and Foulds topological distances to the best tree are rather high, but this has to be interpreted carefully. Indeed, increasing the number of taxa increases the chance of having one or more taxa which position in the tree is hard to determine. Yet, misplacing a single taxon can drastically increase the Robinson and Foulds distance. Altogether, the results with large data sets are thus in good accordance with those obtained with medium data sets, though the small number of alignments (10+10 instead of 50+50) reduces the strength of some comparisons.

Results of RAxML and FastTree using the CAT approximation (Supplementary material), show that, as expected, resulting trees are not as good as those of PhyML ‘SPR’ regarding likelihood values; (NNI-based) FastTree performance is slightly behind that of PhyML 3.0 ‘NNI’, while (SPR-based) RAxML with CAT is in between PhyML 3.0 ‘NNI’ and ‘SPR’. Note however that, with nucleotide sequences, RAxML and FastTree (both with the CAT option) are ~6 times faster than PhyML ‘SPR’ using the full Γ 4 model. With amino-acid data sets FastTree is ~4 time faster than both PhyML ‘SPR’ and RAxML with CAT, these last two showing similar computing times. We thus believe that the use of the CAT approximation should be reserved to exploratory studies or very large data sets.

Computing times. Computing time is also an important factor when comparing methods dealing with large data sets. These times were measured for each alignment and each method. All programs were run on a cluster Intel(R) Xeon(R) CPU 5140 @ 2.33GHz, 24 computing nodes, with 8GB of RAM for one bi-dual-core unit. Only effective CPU times were measured to obtain comparable computing times. Figure 1 reports the base-2 logarithm of the ratio between these values and the corresponding computing times obtained with the fastest approach. Thus, a log-ratio equals to X corresponds to a method being 2^X times slower than the fastest approach.

For medium-size DNA alignments, the original version of 'NNI' and RAxML display similar speeds and are the fastest methods. The new version of 'NNI' is twice as slow as the original release when considering the median of the time ratios. When considering the mean, the new version is only ~1.4 times slower, which illustrates the fact that, for a few data sets, the original version of 'NNI' is significantly slower than the new algorithm. 'SPR' is approximately 4 times slower than the fastest methods while 'BEST' is roughly 1.4 times slower on average than 'SPR'. As expected, 'RAND' is about 6 times slower than 'SPR'. Indeed, five SPR searches with a random starting tree, plus one SPR search with a BioNJ starting tree, are performed for each data set, which explains the multiplicative time factor compared to 'SPR' (one NNI search is also performed, but the computing time is negligible compared to the 6 SPR searches).

The situation is different when looking at medium-size protein alignments. 'SPR' and both versions of 'NNI' have similar computing times and are the fastest methods. RAxML is here noticeably slower than 'SPR' (~4 times), which contrasts with its excellent performance on DNA alignments. This difference is mostly explained by the combination of two factors: (1) 'SPR' does not perform any additional rounds without parsimony-based filtering, as it does with DNA alignments (see above); (2) RAxML implementation is highly optimized for DNA. Finally, as expected, 'BEST' is twice slower than 'SPR' while 'RAND' is ~7 times slower than 'SPR'.

The computing times for large data sets define essentially the same trends. For DNA alignments, the new release of 'NNI' is twice as slow as the original one and 'SPR' is approximately three times slower than RAxML. As for protein alignments, 'SPR' is generally the fastest and RAxML is approximately five times slower.

Summary. Simulated data do not allow for a relevant ranking of compared methods and options, and more informative results are obtained with our large-scale benchmarks comprising 60

DNA and 60 protein alignments of various sizes. Overall, the new version of 'NNI' provides trees with higher likelihoods compared to the original algorithm. However, this comes at the expense of increased computing times with DNA alignments, and both NNI-based algorithms are outperformed in terms of likelihood optimization by SPR-based ones. RAxML is very fast on DNA data sets, but it returns trees that are, on average, slightly less likely than those estimated using 'SPR'. For proteins, RAxML is much slower than 'SPR' but it generally estimates phylogenies with greater likelihoods. Finally, for both DNA and protein alignments, 'RAND' is rather slow but is clearly the most efficient method to maximize the likelihood. All these results, including the alignments, the estimated trees, the computing times and log-likelihoods for the different methods are available from the Supplementary Material web page.

PART II: BRANCH TESTING

Methods

The aLRT statistic. PhyML 3.0 implements a fast approximate likelihood ratio test (aLRT) for branches (Anisimova and Gascuel, 2006), which is a useful complement to the (time-consuming) bootstrap analysis (Felsenstein, 1985). The aLRT is closely related to the conventional LRT, with the null hypothesis that the tested branch has length zero (Felsenstein, 1988). The standard LRT uses the statistic $2(\text{LNL1} - \text{LNL0})$, where LNL1 is the log-likelihood of the best ML tree (denoted as T1), and LNL0 is the log-likelihood of the same tree, but with the branch of interest collapsed to 0 (denoted as T0). The aLRT uses a different (but related) test statistic, which is $2(\text{LNL1} - \text{LNL2})$, where LNL2 corresponds to the second best NNI configuration around the branch of interest (denoted as T2). Computing this statistic with PhyML is fast, as the data structures are optimized for NNI calculations, and because the log-likelihood value LNL2 is computed by optimizing only the branch of interest and the four adjacent branches (as done in refined NNIs with 5-branch-length estimation, see above), while other parameters are fixed at their optimal values corresponding to the best ML tree (T1).

The inequality $\text{LNL2} \geq \text{LNL0}$ holds because T2 and T0 are nested, and often $\text{LNL2} \approx \text{LNL0}$, as the branch of interest in T2 tends to be very short. Using the aLRT statistic (instead of the standard LRT statistic) has the advantage that the test does not support the branch of interest in T1 when the alternative configuration in T2 has a similar likelihood with a significantly positive length of the branch under consideration. Such an event is rare, but was reported by several authors and is

considered as the main pitfall of the standard null-branch test (Felsenstein, 2003, pp. 319-320). Moreover, because $LNL2 \approx LNL0$ in most cases under the null hypothesis, we can use the null distribution of the standard LRT statistic to estimate the confidence region of the approximate test. Since $2(LNL1 - LNL2) \leq 2(LNL1 - LNL0)$ and the two statistics are very close, such an approximation is accurate with a slight conservative bias. Otah et al. (2000) showed that under the null hypothesis the LRT statistic is distributed as a mixture of chi-square distributions: $\frac{1}{2}\chi_1^2 + \frac{1}{2}\chi_0^2$. Moreover, we showed that this distribution has to be combined with a Bonferroni correction, because in practice T1 is not fixed a priori but selected among the three possible NNI configurations. While testing whether a branch length is significantly positive (standard LRT) does not answer the question about the best-fit topological configuration, the aLRT achieves that goal: the chi-square-based interpretation of the aLRT statistic is related to the standard bootstrap from that point of view and was shown to be accurate and powerful with simulated data (Anisimova and Gascuel, 2006). Nevertheless, since real and simulated data differ, for some biological data sets we expect serious violations of the substitution model assumptions, which may perturb the parametric chi-square-based interpretation of the aLRT statistic.

A non-parametric interpretation of the aLRT statistic. To correct for model violations, PhyML 3.0 implements a non-parametric branch-support measure, in line with the SH tree-selection method (Shimodaira and Hasegawa, 1999). The standard SH approach computes a confidence set for an *a priori* given testing set of topologies, which should contain every topology that may be considered as being the true topology. The most likely topology (from the testing set) is selected using the same data set that is used to perform the test. This selection induces some bias which is alleviated by the SH procedure (see Goldman et al., 2000, for explanations). Interpreting the aLRT statistic $2(LNL1 - LNL2)$, i.e. measuring the significance of the difference between T1 and T2, is a closely related task, except that not only T1 but also T2 are selected using the same data set. We thus implemented a variant of the standard SH procedure, using RELI bootstrap (Kishino and Hasegawa, 1989).

Let $sLNL1$, $sLNL2$ and $sLNL3$ be the sets of log-likelihood values for all sites of the alignment assuming T1, T2 and T3 (the third NNI configuration), respectively, and $LNL1$, $LNL2$ and $LNL3$ be equal to the sums of $sLNL1$, $sLNL2$ and $sLNL3$ entries, respectively. Let $sLNL1^*$, $sLNL2^*$ and $sLNL3^*$ be the site log-likelihood samples obtained using RELI bootstrap from $sLNL1$, $sLNL2$ and $sLNL3$, respectively. As usual with RELI, this involves drawing sites with replacement and using the same set of sites for each of the three $sLNLX^*$ bootstrap samples. Let $LNL1^*$, $LNL2^*$ and $LNL3^*$

be the sums of $sLNL1^*$, $sLNL2^*$ and $sLNL3^*$ entries, respectively. Note that the expectation of any $LNLX^*$ is equal to $LNLX$ due to bootstrap sampling properties. Our SH-like algorithm to estimate the confidence of the aLRT statistics is summarized in Appendix C.

This algorithm is a natural adaptation of the SH procedure, as described by Goldman et al. (2000). It simulates the distribution of the aLRT statistic under the null hypothesis that all T1, T2 and T3 configurations are equally likely. This assumption is expressed in the centering step C that makes equal (and null) the expectations of the three CSX^* variables, from which the test distribution is computed. Moreover, the variances and covariances of the CSX^* are the same as those of the $LNLX^*$, which simulate the variability of $LNL1$, $LNL2$ and $LNL3$ thanks to bootstrap sampling. Steps D and E mimic the selection of T1 (best configuration) and T2 (second best configuration), which is at the core of the aLRT statistic. The difference with the standard SH procedure lies in these two steps (D, E), where we compute the support for a specific branch of the ML tree (T1), instead of computing the separate supports of T1, T2 and T3. We thus obtain a unique branch support instead of 3 confidence values that are difficult to combine into a single summary value. However, experiments show that (as expected) our SH-like branch support is relatively close to 1 minus the standard SH support of T2. Small ϵ in step E (0.1 in our current implementation) is used to avoid rounding effects, for example when both T1 and T2 correspond to very short branches and have nearly identical site log-likelihood values.

Computation of branch supports using this algorithm (Appendix C) is very fast, as we simply draw and sum values stored in an appropriate array. In fact, computing the aLRT statistics of all branches and interpreting these statistics using either the chi-square-based or the SH-like procedures has a negligible computational cost in comparison with tree building. Actually, all time consuming computations needed for that test are already done in refined NNIs with 5-branch-length estimation. This contrasts with standard bootstrap, which increases the computing time by a factor of 100 – 1,000, depending on the number of bootstrap samples required by the user. The rapid bootstrap approach proposed by Stamatakis et al. (2008) is faster than standard bootstrap thanks to simplified SPR tree search with bootstrap samples, but is still much slower than aLRT branch testing.

Results

Comparisons of aLRT and bootstrap supports with simulated data. We used our 100 40-taxon simulated data sets (see above) to compare Felsenstein's non-parametric bootstrap and aLRT with

both chi-square-based and SH-like branch supports. As with simulated data we know the true tree used to generate the sequences, the aim was to check whether each method provides high supports to correct branches and low supports to incorrect ones, that is, branches that do not belong to the true tree. Note, however, that very short branches sometimes are not supported by any substitution; for example, with 500 sites (as used in this study) any branch of length $<0.002 = 1/500$ has low chance to encounter even a single substitution. In this case the branch is still “correct” as it belongs to the true tree, but any branch testing method should provide a very low support for that branch. In our simulated data sets, ~5% of the branches correspond to this situation and are not supported by any substitution. Moreover, with real data the substitution model is unknown and most likely more complex than the standard Markov models used in tree building and branch testing. To check method robustness and assess the impact of this gap between the true substitution process and the model actually used, we inferred the trees and tested the branches using the Jukes and Cantor model (1969) with no gamma distribution of site rates. This model (JC69) is oversimplified compared to the GTR+ Γ 4 model used to generate the data; it severely violates a number of features of our simulated sequences as it ignores the variations of rates across sites, the unequal base frequencies, and the differences between relative rates for each type of substitution. To some extent, using JC69 to analyze our GTR+ Γ 4 data thus reproduces the simplification that is inherent in any analysis of real data.

Results are displayed in Figure 2. Let us consider the standard statistical interpretation of branch supports (e.g. Felsenstein, 2003, pp. 346-357). These are viewed as being equal to 1 minus the p -value of a test which null hypothesis basically means that the branch of interest is incorrect. When the p -value is smaller than a given significance level, typically 0.05 (the support is then larger than 0.95), the null hypothesis is rejected and the branch is considered to be correct; otherwise, the support is less than 0.95 and the branch is deemed to be incorrect. In that perspective, aLRT with chi-square-based interpretation performs very well when the true model (GTR+ Γ 4) is used to analyze the data. Indeed (see Fig. 2(a)), with a significance level equal to 0.1, exactly 90% of incorrect branches are predicted to be incorrect and 10% to be correct. In other words, the obtained and desired Type-1 errors are equal. As a consequence, the test is powerful and retains most of correct branches (~90%, Fig. 2(b)). In this condition and using the same significance level (0.1), both bootstrap and aLRT with SH-like supports are accurate but conservative; only ~1% of incorrect branches are predicted to be correct and, consequently, both tests are not powerful and reject a significant proportion of correct branches (38% and 35% for bootstrap and aLRT SH-like supports, respectively).

Actually, we do not expect real data to fit any evolutionary model perfectly, and simulations with model violations are likely to be more realistic than most simulation setups, in which the true and estimated substitution models are identical. When JC69 is used to analyze the (GTR+ Γ 4) data, aLRT with chi-square-based supports is no more accurate (Fig. 2(a)); with 0.1 significance level, up to 30% incorrect branches are predicted to be correct. On the opposite, both bootstrap and aLRT SH-like supports are still accurate, though conservative. Both are not much affected by the JC69 model violation, as expected due their non-parametric nature, while aLRT with chi-square-based supports performs very well when its parametric assumptions are fulfilled (GTR+ Γ 4 analyses), but not so in the more realistic case. Altogether, these results suggest using aLRT with SH-like (rather than chi-square-based) supports, though this approach is expected to be somewhat conservative.

Statistical interpretation of bootstrap supports has been the subject of an intense debate since the 90's (see Felsenstein, 2003, pp. 346-357), with no consensus reached. Currently, the common practice is to use "a rule of thumb" whereby sufficient evidence is indicated by bootstrap supports above 0.7-0.8. Results in Figure 2(a) validate this practice, as such a selection threshold discards most of the incorrect branches. On the contrary, aLRT is a standard statistical test and its interpretation is unambiguous: a p -value is computed and compared to some significance level to decide which branches are retained and which ones should be discarded. However, as aLRT SH-like supports tend to be conservative, we can use an empirical rule, just as with bootstrap. Based on Figure 2(a), the selection threshold for SH-like supports should be in the 0.8-0.9 range. In our analyses with JC69, using 0.75 and 0.85 as thresholds of branch selection for bootstrap and aLRT SH-like supports respectively, both methods have similar power (~85% of correct branches are selected).

Finally, we see from Figure 2(a) that relatively high supports are often given to incorrect branches (with JC69 analyses, both bootstrap and aLRT SH-like median support values are close to 0.5). Similarly, we see from Figure 2(b) that "correct" branches with no substitution (i.e. ~5% of correct branches) often have high supports, specially with bootstrap where only 1% of correct branches have support <0.3. These observations indicate that medium support values (say, around 0.5) should not be considered as supporting the presence of the given branch in the true tree.

Comparisons of aLRT and bootstrap supports with real data. We also compared standard bootstrap to aLRT using our medium-size data sets (50 protein and 50 DNA alignments, see above). Figure 3 displays the plots obtained with two representative alignments (detailed results and graphics

for all data sets are available in Supplementary Material). Note that neither chi-square-based aLRT branch supports nor SH-like supports are expected to be equal to bootstrap supports (not even approximately). As discussed above, statistical interpretation of bootstrap is still a subject of debate, while both aLRT supports are closely related to p -values of statistical tests.

Figure 3(a) shows branch supports for a protein alignment of 22 taxa and 513 sites, where bootstrap (BP) and aLRT with SH-like (SH) supports clearly agree. All branches with $BP > 0.75$ have $SH > 0.90$, and all branches but one (among 11) with $SH > 0.90$ have $BP > 0.75$. In other words, both methods support nearly the same set of branches when using standard moderate selection thresholds. Moreover, it is clear from this figure and other analyses (Fig. 3(b) and Supplementary Material) that SH-like supports are more conservative than chi-square-based supports and closer to the main bootstrap tendencies. As explained above, this likely stems from the non-parametric nature of both SH and bootstrap procedures.

However, not all data sets show such high level of congruence. Both approaches, aLRT with SH-like interpretation and standard bootstrap, tend to agree for data sets with strong phylogenetic signal, that is, with sufficiently long and sufficiently diverged sequences resulting from a homogeneous, quasi-Markov substitution process. Disagreements in branch supports arise as a consequence of small samples, insufficient or saturated divergence levels, and are more likely if the substitution process is highly heterogeneous. For example, Figure 3(b) shows branch supports for a DNA alignment of 68 taxa with 1,195 highly gapped and poorly informative sites (only 335 sites have less than 10% gaps, and 526 do not show any polymorphism); moreover, the corresponding tree contains a number of very short internal branches (21 among 65 have zero length, and the median value of internal branch lengths is ~ 0.02). The agreement level is clearly lower in comparison with the previous data set (Fig. 3(a)) that contains more informative sites (510 sites have less than 10% gaps, and 86 sites only have no polymorphism) and shows higher sequence divergence (no branch has zero length and the median value of internal branch lengths is ~ 0.06).

One striking example of support disagreement in Figure 3(b) is a branch with $BP = 0.08$ and SH-like support in the 0.90-0.95 range. In this case, it is likely that the aLRT is overconfident due to the limited number of topological configurations considered when estimating the branch statistics. Indeed, the aLRT assesses whether the branch being studied in T1 provides a significant gain in likelihood in comparison with the alternative NNI resolutions T2 and T3, while the rest of the tree remains intact.

Thus, the aLRT does not account for other possible topologies that may be highly likely but quite different from the inferred topology. This implies that the aLRT performs well when the data contains a clear phylogenetic signal and a good ML topology has been found, but not as well in the opposite case, where it tends to give a too local view focusing on the branch of interest and ignoring the possible effects of some other parts of the tree.

Figure 3(b) also exhibits branches with aLRT statistics nearly equal to 0, which have medium to high bootstrap supports (up to 0.84). Most of these branches have lengths very close or equal to 0.0, which means that they are not supported by even a single substitution. Thus, the standard bootstrap is faced with the paradox of supporting branches without signal in the data. To check that this phenomenon is not specific to PhyML, we ran other programs and observed similar behaviors; for example, the tendency to obtain high bootstrap supports for very short branches is more prominent with RAxML-based fast bootstrap (Stamatakis et al., 2008) than with PhyML (see Supplementary Material). An explanation could be the so-called “star paradox”, which indicates that even in the absence of a signal (the star tree), we expect high supports for some branches (Steel and Matsen, 2007; Susko, 2008). However, the phenomenon is so strong in some cases (e.g. Fig. 3(b) and RAxML results in Supplementary Material) that other factors likely contribute. Hidden determinisms could play a major part. While some choices should be purely random in the absence of signal, they are actually not random at all, and due to program implementation the same choices are made for each bootstrap sample. Pair agglomeration in BioNJ (used by PhyML to initiate the tree search) is an example of such choice, which should be random when some sequences are (nearly) identical. A simple and efficient trick, used in PhyML 3.0 and most of PHYLIP programs, is to jumble taxon ordering before analyzing each of the bootstrap samples. However, some other sources of hidden determinism likely remain in PhyML 3.0 (and most if not all phylogenetic programs). Thus, a practical solution is to combine aLRT and bootstrap supports, as the two are likely to compensate for each other’s failures: while aLRT does not support extremely short branches, the bootstrap procedure uses a fairer sample of the topology space and is not biased towards the ML tree inferred from the original data (unlike aLRT, which is limited to the NNI rearrangements of this ML tree).

Figure 4 illustrates that the agreement between standard bootstrap and aLRT SH-like supports increases as the phylogenetic signal becomes stronger. The agreement between SH and BP is measured by the ratio $|\text{SH}_{90} \cap \text{BP}_{75}| / |\text{SH}_{90} \cup \text{BP}_{75}|$, where SH_{90} is the set of branches with $\text{SH} > 0.90$, BP_{75} is the set of branches with $\text{BP} > 0.75$, and $|S|$ denotes the size of set S . This ratio is 1

when both methods support the same set of branches, and is 0 when they fully disagree. The strength of the phylogenetic signal is measured by the product of the number of sites by the median value of internal branch lengths; only sites with less than 10% gaps and missing values are accounted for, to avoid comparing fully gapped, uninformative sites and complete sites. This measure of phylogenetic signal approximately corresponds to the expected number of substitutions per branch, and is close to the tree-length-based criterion used in Anisimova et al. (2001). For example, a value of 1 indicates a low information content as the average expected number of substitutions per branch over the whole sequence is only 1, and it is likely that the shortest branches are not supported by any substitution. In Figure 4 we plotted the SH vs. BP agreement ratio as a function of phylogenetic signal for our 50 DNA data sets and for the 30 protein data sets comprising at least 20 taxa (with less taxa the agreement ratio tends to be poorly estimated). Strong agreement between SH and BP supports is observed when the phylogenetic signal is sufficiently high; for example, when the signal is larger than 10, all trees but one have agreement > 0.5 with an average of 0.77 for DNA data sets and 0.76 for protein ones. With our two example alignments of Figure 3, the (agreement, signal) pair is equal to (0.91, 32.3) and (0.75, 6.9) for 3(a) and 3(b), respectively.

Summary. Experiments with simulated data indicate that the new SH-like interpretation of the aLRT statistic should be preferred to the parametric Chi-square-based interpretation, due to unavoidable simplifications of substitution models when analyzing real data. Moreover, both aLRT with SH-like interpretation and standard bootstrap are conservative. Experiments with 50 DNA and 50 protein data alignments show that both aLRT with SH-like interpretation and standard bootstrap tend to agree for informative data, but both have their own limitations when the phylogenetic signal is weak. In such cases, all support values need to be considered with caution. We recommend combining the two supports, as SH-like aLRT is robust for short branches (unlike bootstrap), and because bootstrap supports are based on a better sample of topologies than the NNI-based configurations used in the aLRT statistic. In exploratory stages, with large data sets or limited computational resources, the aLRT with SH-like interpretation provides valuable information with sufficient accuracy and is very fast.

AVAILABILITY

PhyML web server is accessible at <http://www.atgc-montpellier.fr/phyml/>. Executable files for a variety of computer architectures and a manual can be downloaded free of charge at this URL. The

sources are also available upon request to guindon@lirmm.fr. The program is written in C and its compilation is usually straightforward. A version of the program based on the MPI library allows conducting bootstrap analyses in parallel, which potentially saves considerable amounts of computing times. The web server also allows users to upload their own data sets. The alignments are then processed on our server at LIRMM (Montpellier). This server is an Intel(R) Xeon(R) CPU 5140 @ 2.33GHz, 24 computing nodes, with 8GB of RAM for one bi-dualcore unit, which allows the processing of fairly large and numerous data sets in short amounts of time. Moreover, we plan to use Grid computing facilities in the near future. Once the execution is finished, the results are sent back to the user via an electronic message, which includes a link to a web page displaying the estimated tree using the ATV Java applet (Zmasek and Eddy, 2001).

CONCLUSION

PhyML 3.0 implements new algorithms to search the space of tree topologies with user-defined intensities. The analysis of real-world DNA and protein sequence data sets shows that these options are useful to quickly search the tree space in exploratory stages (using NNIs), and to perform intensive topology searches when all parameters of the study (taxon sampling, alignment, evolutionary model, etc.) are fixed (using the 'RAND' option). PhyML 3.0 also implements a non-parametric, Shimodaira-Hasegawa-like branch test, which is a very fast alternative and complement to the standard bootstrap analysis. Moreover, several new evolutionary models are provided, and the interface was entirely re-designed. We believe that PhyML 3.0 is now stable and ready-to-use. A web server and binaries are available from PhyML web page.

Further developments will include: (1) mixture and partitions models, for example allowing for different models for the three codon positions, accounting for the structure of proteins (e.g. Le and Gascuel, 2010), or dealing with multi-gene studies (e.g. Pagel and Meade, 2005); (2) constrained topological searches, for example accounting for the partial knowledge of the phylogeny, building the in-group and out-group trees separately before merging into the final phylogeny, or, simply, inserting new species in a well established phylogenetic tree. Finally, we plan to distribute our benchmark data sets and comparison programs using a web server, which will allow phylogeny software developers to compare their algorithms with standardized methods.

REFERENCES

- Anisimova, M., J.P. Bielawski, and Z. Yang. 2001. Accuracy and power of the likelihood ratio test in detecting adaptive molecular evolution. *Mol. Biol. Evol.* 18:1585-92.
- Anisimova, M., and O. Gascuel. 2006. Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. *Syst. Biol.* 55:539-52.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783-791.
- Felsenstein, J. 1988. Phylogenies from molecular sequences: inference and reliability. *Annu. Rev. Genet.* 22:521-565.
- Felsenstein, J. 2003. *Inferring phylogenies*. Sinauer Associates, Inc., Sunderland, MA.
- Gascuel, O. 1997. BIONJ: An improved version of the NJ algorithm based on a simple model of sequence data. *Mol. Biol. Evol.* 14:685-695.
- Goldman, N., J.P. Anderson, and A.G. Rodrigo. 2000. Likelihood-based tests of topologies in phylogenetics. *Syst. Biol.* 49:652-670.
- Guindon, S., and O. Gascuel. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* 52:696-704.
- Hordijk, W., and O. Gascuel. 2005. Improving the efficiency of SPR moves in phylogenetic tree search methods based on maximum likelihood. *Bioinformatics* 21:4338-4347.
- Jobb, G., A. von Haeseler, and K. Strimmer. 2004. TREEFINDER: A powerful graphical analysis environment for molecular phylogenetics. *BMC Evol. Biol.* 4:18.
- Jukes, T., and C. Cantor. 1969. Evolution of protein molecules. Pages 21-132 *in* *Mammalian Protein Metabolism*, Volume III, Chapter 24 (H. Munro, ed.). Academic Press, New York.
- Kishino, H., and M. Hasegawa. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J. Mol. Evol.* 29:170-179.
- Kuhner, M.K., and J. Felsenstein. 1994. A simulation comparison of phylogeny algorithms under equal and unequal evolutionary rates. *Mol. Biol. Evol.* 11:459-468.

- Lanave, C., G. Preparata, C. Saccone, and G. Serio. 1984. A new method for calculating evolutionary substitution rates. *J. Mol. Evol.* 20:86–93.
- Le, S.Q., and O. Gascuel. 2008. An improved general amino acid replacement matrix. *Mol. Biol. Evol.* 25:1307-1320.
- Le, S.Q., and O. Gascuel. 2010. Accounting for solvent accessibility and secondary structure in protein phylogenetics is clearly beneficial. *Syst. Biol.*, in press.
- Lemmon, A., and M.C. Milinkovitch. 2002. The metapopulation genetic algorithm: an efficient solution for the problem of large phylogeny estimation. *Proc. Natl. Acad. Sci. U.S.A.* 99:10516-10521.
- Olsen, G.J., H. Matsuda, R. Hagstrom, and R. Overbeek. 1994. fastDNAm1: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput. Appl. Biosci.* 10:41-1048.
- Ota, R., P.J. Waddell, M. Hasegawa, H. Shimodaira, and H. Kishino. 2000. Appropriate likelihood ratio tests and marginal distributions for evolutionary tree models with constraints on parameters. *Mol. Biol. Evol.* 17:798-803.
- Pagel, M., and A. Meade. 2005. Mixture models in phylogenetic inference. Pages 121-142 *in* *Mathematics of Evolution & Phylogeny* (O. Gascuel, ed.). Oxford University Press, Oxford.
- Penny, D., and M. Steel. 2000. Parsimony, likelihood, and the role of models in molecular phylogenetics. *Mol. Biol. Evol.* 17:839-50.
- Price, M., Dehal, P., Arkin, A. 2009. FastTree 2.1 – Approximately maximum-likelihood trees for large alignments. <http://www.microbesonline.org/fasttree/ft2-Oct15.pdf>
- Ranwez, V., and O. Gascuel. 2001. Quartet-based phylogenetic inference: improvements and limits. *Mol. Biol. Evol.* 18:1103-11016.
- Ronquist, F., and J.P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572-1574.
- Sanderson, M.J., M.J. Donoghue, W. Piel, and T. Eriksson. 1994. TreeBASE: a prototype database of phylogenetic analyses and an interactive tool for browsing the phylogeny of life. *Amer. Jour. Bot.* 81:183. <http://www.treebase.org/>

- Shimodaira, H., and M. Hasegawa. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* 16:1114–1116.
- Stamatakis, A. 2006a. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688-2690.
- Stamatakis, A. 2006b. Phylogenetic models of rate heterogeneity: a high performance computing perspective. In the Proceedings of the 20th IEEE/ACM International Parallel and Distributed Processing Symposium (IPDPS 2006).
- Stamatakis, A., P. Hoover, and J. Rougemont. 2008. A rapid bootstrap algorithm for the RAxML Web servers. *Syst. Biol.* 57:758-71.
- Steel, M., and F.A. Matsen. 2007. The Bayesian "star paradox" persists for long finite sequences. *Mol. Biol. Evol.* 24:1075-9.
- Susko, E. 2008. On the distributions of bootstrap support and posterior distributions for a star tree. *Syst. Biol.* 57:602-12.
- Vinh, S., and A. von Haeseler. 2004. IQPNNI: moving fast through tree space and stopping in time. *Mol. Biol. Evol.* 21:1565–1571.
- Whelan, S. 2007. New approaches to phylogenetic tree search and their application to large numbers of protein alignments. *Syst. Biol.* 56:727-740.
- Whelan, S., and N. Goldman. 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* 18:691-699.
- Yang, Z. 1993. Maximum-likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. *Mol. Biol. Evol.* 10:1396–1401.
- Zmasek, C.M., and S.R. Eddy. 2001. ATV: display and manipulation of annotated phylogenetic trees. *Bioinformatics* 17:383-384.
- Zwickl, D.J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence data sets under the maximum likelihood criterion. Ph.D. dissertation, The University of Texas at Austin.

APPENDIX A: ONE SPR CYCLE

ALGORITHM: One_Spr_Cycle

Input: tree T.

Output: tree T with improved likelihood.

Let PARS and LNL be the parsimony and log-likelihood of T.

For each subtree S in tree T, do {

- A. Clear SPR_List, the list of SPR moves;
- B. Prune S;
- C. For each possible regraft position for S {
 1. Calculate PARS', the parsimony score of the new tree T' ;
 2. Store PARS' in SPR_List along with the change of tree topology $T \leftarrow T'$;}
- D. Sort SPR_List by increasing values of parsimony scores;
- E. Regraft S at its original position;
- F. Let BEST_LNL' $\leftarrow -\infty$ and BEST_T' \leftarrow NULL;
- G. For the SPR moves in SPR_list with parsimony scores PARS' smaller than PARS+PT (where PT is a predefined parsimony threshold) {
 1. Prune S;
 2. Work out the ML estimate of the branch length at the prune position;
 3. Regraft S (i.e., $T \leftarrow T'$);
 4. Evaluate $LNL' = \ln L(T')$, the log-likelihood of T', without adjusting the three branch lengths at the regraft site (the regraft site is in the middle of the original edge and the pruned edge is left unchanged);
 5. If $LNL' > BEST_LNL'$, then $BEST_LNL' \leftarrow LNL'$, $BEST_T' \leftarrow T'$, go to G;
 6. Else, {
 - i. Work out the three ML branch length estimates at the regraft position and calculate $LNL' = \ln L(T')$;
 - ii. If $LNL' > BEST_LNL'$, then $BEST_LNL' \leftarrow LNL'$, $BEST_T' \leftarrow T'$, go to G;}}
- H. If $BEST_LNL' > LNL$, then $T \leftarrow BEST_T'$, $LNL \leftarrow BEST_LNL'$, $PARS \leftarrow PARS'$
- I. Update the partial-parsimony and partial-likelihood for every subtree in T;

}
Return T

APPENDIX B: MULTIPLE SPR CYCLES

ALGORITHM: Multiple_Spr_Cycles

Input: tree T

Output: tree T with improved likelihood

Let LNL be the likelihood of T.

$LNL' \leftarrow LNL - 2\epsilon$;

While $(LNL - LNL' > \epsilon)$ {

A. $LNL' \leftarrow LNL$;

B. $T \leftarrow \text{One_Spr_Cycle}(T)$;

C. Adjust the parameters of the substitution model;

D. Adjust branch lengths;

E. Update the likelihood of T: $LNL = \ln L(T)$;

}

Return T

APPENDIX C: SH-LIKE INTERPRETATION OF ALRT STATISTIC

ALGORITHM: SH-like interpretation of aLRT statistic

Input: lists of site log-likelihood values sLNL1, sLNL2, sLNL3

Output: branch-support SH

$aLRT \leftarrow 2(LNL1 - LNL2)$;

$SH \leftarrow 0$;

For $i=1$ to ReplicateNumber (typically 1000): {

A. Draw $sLNL1^*$, $sLNL2^*$ and $sLNL3^*$;

B. Compute $LNL1^*$, $LNL2^*$ and $LNL3^*$;

C. Compute centered sums $CS1^*$, $CS2^*$ and $CS3^*$, using

$CSX^* \leftarrow LNLX^* - LNLX$;

D. Let CS_First and CS_second be the highest and second highest CSX^* values, respectively;

E. If $aLRT > 2(CS_First - CS_Second) + \epsilon$, then $SH \leftarrow SH+1$; }

Return $SH/\text{ReplicateNumber}$

	Av. LogLk rank	Delta>5	p -value<0.05	Av. RF distance
DNA				
PhyML-SPR	3,49	15	1	0.24
PhyML 3.0 SPR (PT=0)	2,43	3	0	0.09
PhyML 3.0 SPR (PT=5)	2,17	2	0	0.06
PhyML 3.0 SPR (PT= ∞)	1,91	2	0	0.05
Protein				
PhyML SPR	2,85	7	2	0.18
PhyML 3.0 SPR (PT=0)	2,69	2	0	0.12
PhyML 3.0 SPR (PT=5)	2,25	2	0	0.06
PhyML 3.0 SPR (PT= ∞)	2,12	1	0	0.04

TABLE 1. Performance of the parsimony filter. PhyML-SPR filter (Hordijk and Gascuel, 2005) uses distance-based minimum evolution principle, while PhyML 3.0 SPR filter uses parsimony. When $PT=\infty$ all SPRs are evaluated with likelihood, without any preliminary filtering. On the opposite, $PT=0$ corresponds to strong filtering (see text). The column ‘Av. LogLk rank’ gives the average log-likelihood ranks for the different methods. These ranks are corrected by taking into account information on tree topologies (see text). ‘Delta>5’ gives the number of cases (among 50) for which the difference of log-likelihood between the method of interest and the highest log-likelihood for the corresponding data set is greater than 5. The column ‘ p -value<0.05’ displays the number of cases for which the difference of log-likelihood when comparing the method of interest to the corresponding highest log-likelihood is statistically significant (SH test). The ‘Av. RF distance’ values are the average Robinson and Foulds topological distances between the trees estimated by the method of interest and the corresponding most likely trees (0 corresponds to identical trees, while 1 means that the two trees do not have any clade in common).

	Av. LogLk rank	Delta>5	<i>p</i> -value<0.05	Av. RF distance (to true tree)
PhyML 2.4.5 NNI	3.98	4	0	0.102
PhyML 3.0 NNI	3.59	3	0	0.100
PhyML 3.0 SPR	3.71	0	0	0.100
PhyML 3.0 BEST	3.08	0	0	0.097
PhyML 3.0 RAND	2.80	0	0	0.097
RAxML	3.86	0	0	0.097

TABLE 2. Performance of tree searching algorithms on 100 simulated DNA alignments. See legend of Table 1 and text for the various PhyML 3.0 options. Note that, in this table, the Robinson and Foulds distance measures the topological difference between true and inferred trees (instead of the difference between inferred and most likely trees, as for the other tables).

	Av. LogLk rank	Delta>5	p -value<0.05	Av. RF distance
DNA				
PhyML 2.4.5	5.48	34	4	0.30
PhyML 3.0 NNI	5.18	33	5	0.28
PhyML 3.0 SPR	2.78	2	0	0.15
PhyML 3.0 BEST	2.70	2	0	0.15
PhyML 3.0 RAND	1.64	0	0	0.03
RAxML	3.22	3	2	0.20
Protein				
PhyML 2.4.5	5.05	21	1	0.26
PhyML 3.0 NNI	4.33	20	1	0.24
PhyML 3.0 SPR	3.24	5	0	0.14
PhyML 3.0 BEST	3.16	4	0	0.14
PhyML 3.0 RAND	2.35	0	0	0.03
RAxML	2.86	0	0	0.08

TABLE 3. Comparison of log-likelihoods on 50 DNA and 50 protein medium-size data sets. See legend of Table 1 and text for details about the various PhyML 3.0 options.

	Av. LogLk rank	Delta>5	<i>p</i> -value<0.05	Av. RF distance
DNA				
PhyML 2.4.5	3.50	10	8	0.47
PhyML 3.0 NNI	3.50	10	7	0.46
PhyML 3.0 SPR	1.40	3	0	0.15
RAxML	1.60	5	1	0.23
Protein				
PhyML 2.4.5	3.45	7	3	0.24
PhyML 3.0 NNI	2.65	6	3	0.20
PhyML 3.0 SPR	2.75	7	0	0.18
RAxML	1.14	0	0	0.00

TABLE 4. Comparison of log-likelihoods on 10 DNA and 10 protein large data sets. See legend of Table 1 and text for details about the various PhyML 3.0 options.

FIGURE CAPTIONS

FIGURE 1. Distribution of relative computing times. For each of the 4 sets of alignments (50 DNA and 50 protein medium-size alignments plus 10 DNA and 10 protein large-size alignments) we measured the base-2 logarithm of the ratio between the computing time of the given method, and that of the fastest approach with the corresponding alignment. Thus, a log-ratio equals to X corresponds to a method being 2^X times slower than the fastest approach; e.g. with DNA alignments PhyML 2.4.5 NNI is basically twice faster than PhyML 3.0 NNI, but both are pretty much the same with protein alignments.

FIGURE 2. Comparison of branch supports with simulated data. These graphics show the distribution of supports (vertical axis) using boxes and whisker plots with bounds provided on the right of the corresponding panel. GTR+ Γ 4: both data generation and analysis (tree inference and branch testing) are performed with the same model. JC69: data are generated with GTR+ Γ 4, but the analysis is performed using a simple JC69 model; this mimics real data analyses in which the standard substitution models used for estimation inevitably simplify the true evolutionary processes. BP: bootstrap supports; KI2: aLRT with chi-square-based branch supports; SH: aLRT with SH-like branch supports.

FIGURE 3. Comparisons of bootstrap and aLRT supports for two data sets. Horizontal axis: aLRT statistic; vertical axis: bootstrap support. Circle symbols indicate the range of SH-like supports. The 0.9 chi-square-based support threshold is shown with a vertical line. Graphic headers indicate the main features of the alignment, e.g. 3(a): proteins, accession number in Treebase M1499, 22 taxa, 513 sites (510 with less than 10% of gaps or missing values), phylogenetic signal equals to 32.3. The phylogenetic signal is measured by the number of sites (with less than 10% gaps) times the median of internal branch lengths. This roughly corresponds to the expected number of substitutions supporting any given internal branch. See text for further details and explanations.

FIGURE 4. Bootstrap and aLRT-SH agreement as a function of the phylogenetic signal. M1499 and M2588 are the two data sets shown in Figure 3. The phylogenetic signal is measured by the number of sites (with less than 10% gaps or missing values) times the median of internal branch lengths. This roughly corresponds to the expected number of substitutions supporting any given internal branch. Branch support agreement equals the proportion of branches with both SH-like support > 0.90 and bootstrap support > 0.75 . See text for further details and explanations.

FIGURE 1. Distribution of relative computing times

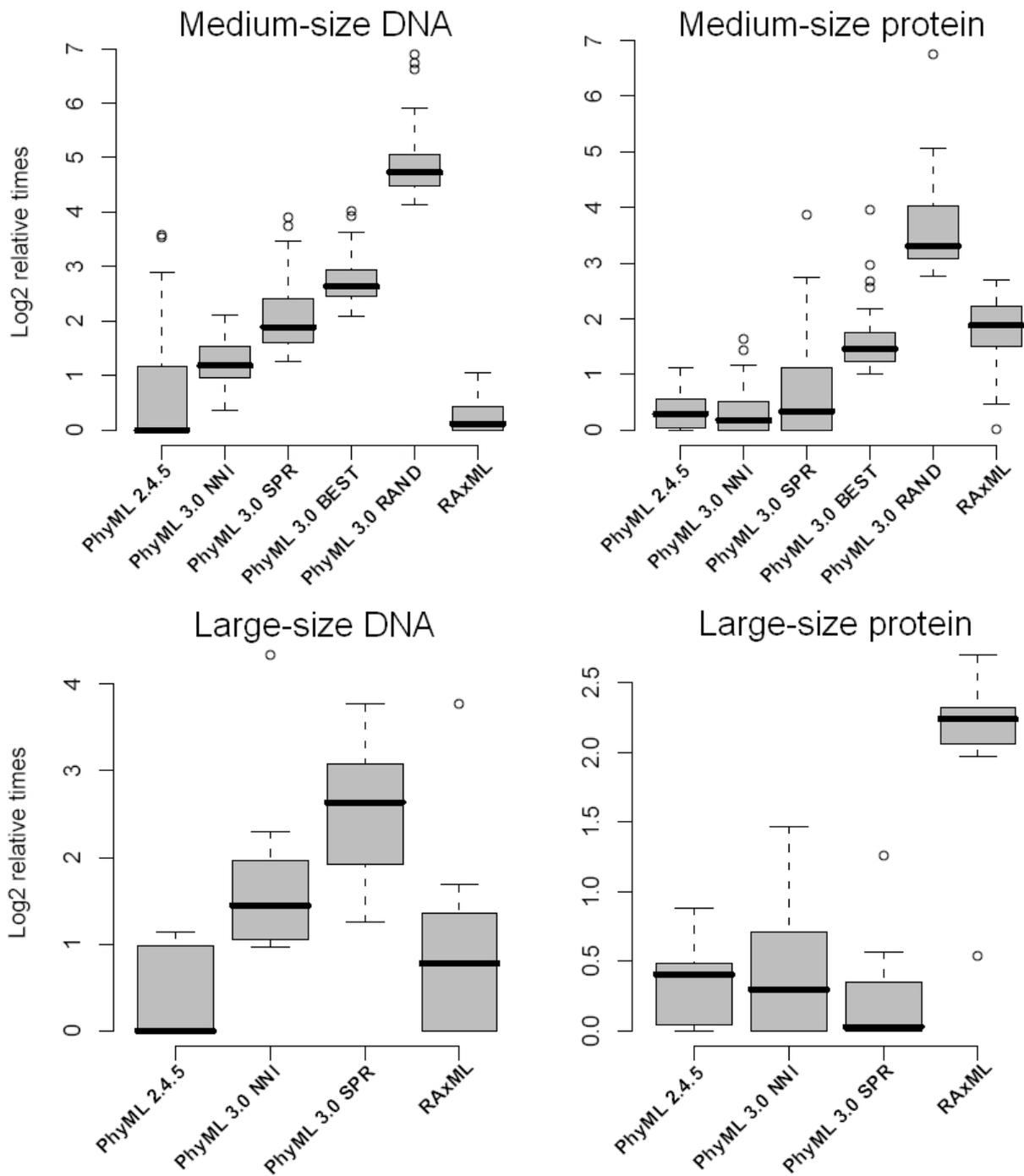


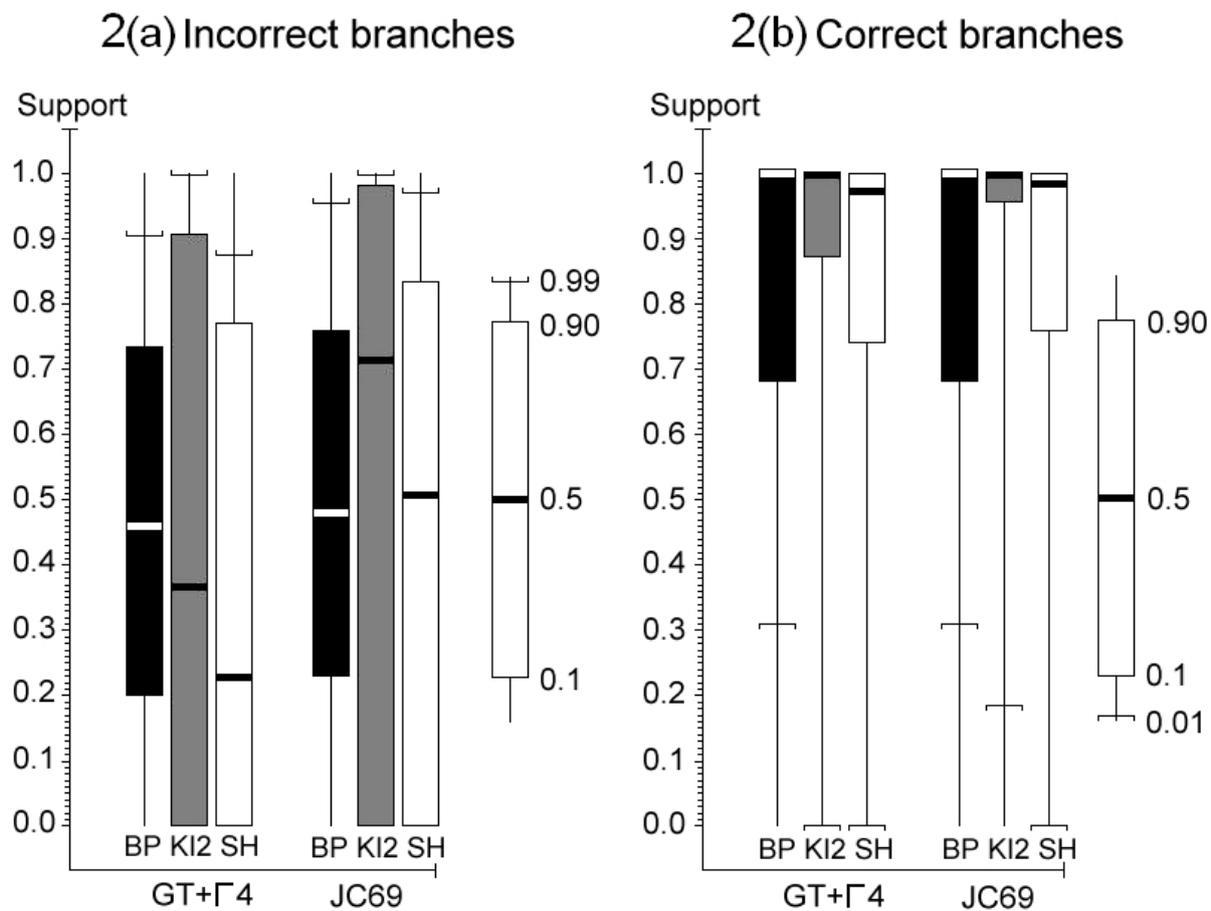
FIGURE 2. Comparison of branch supports with simulated data

FIGURE 3. Comparisons of bootstrap and aLRT supports for two data sets

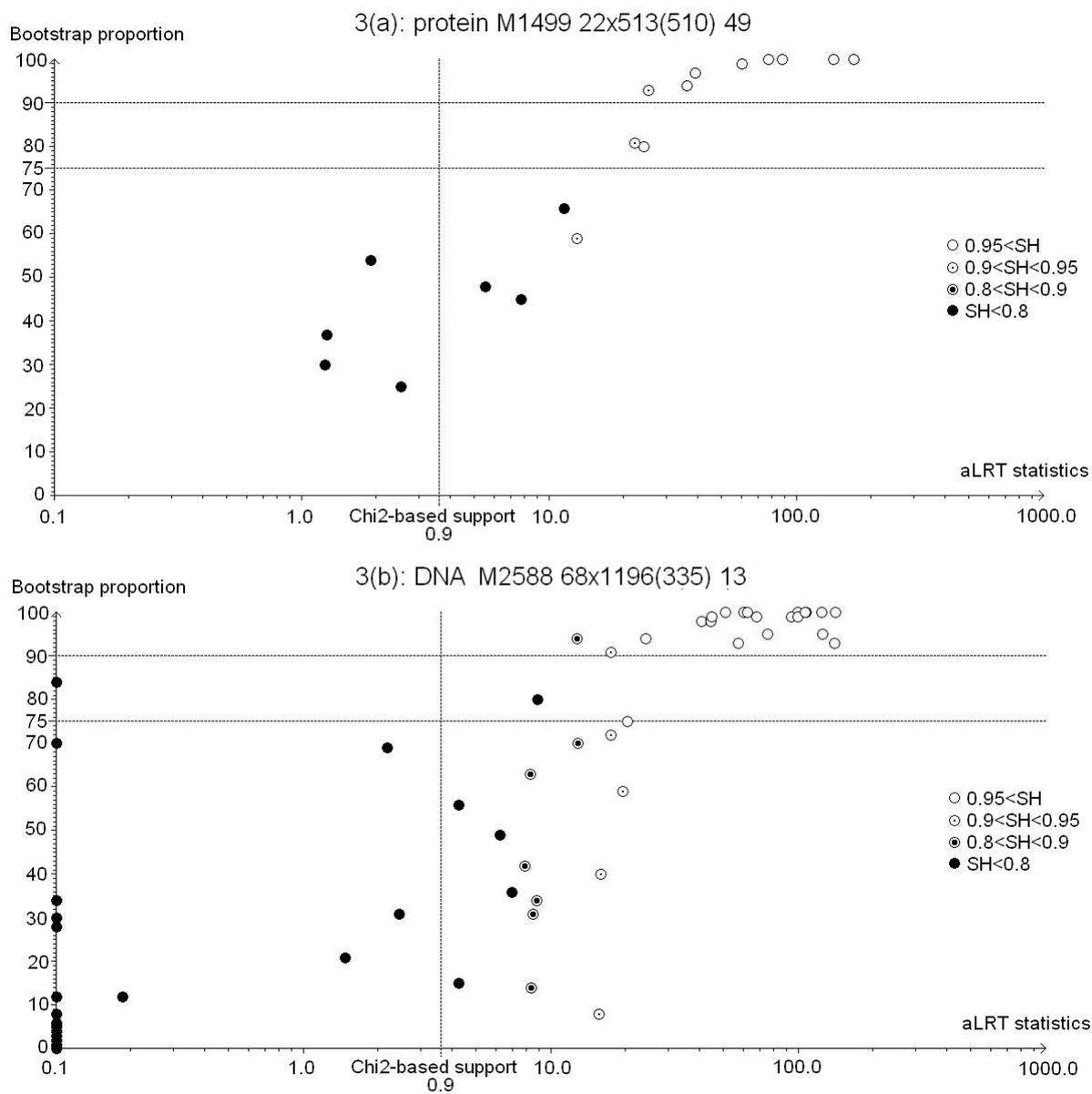


FIGURE 4. Bootstrap and aLRT-SH agreement as a function of the phylogenetic signal

