MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods

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Abstract

Comparative analysis of molecular sequence data is essential for reconstructing the evolutionary histories of species and inferring the nature and extent of selective forces shaping the evolution of genes and species. Here, we announce the release of Molecular Evolutionary Genetics Analysis version 5 (MEGA5), which is a user-friendly software for mining online databases, building sequence alignments and phylogenetic trees, and using methods of evolutionary bioinformatics in basic biology, biomedicine, and evolution. The newest addition in MEGA5 is a collection of maximum likelihood (ML) analyses for inferring evolutionary trees, selecting best-fit substitution models (nucleotide or amino acid), inferring ancestral states and sequences (along with probabilities), and estimating evolutionary rates site-by-site. In computer simulation analyses, ML tree inference algorithms in MEGA5 compared favorably with other software packages in terms of computational efficiency and the accuracy of the estimates of phylogenetic trees, substitution parameters, and rate variation among sites. The MEGA user interface has now been enhanced to be activity driven to make it easier for the use of both beginners and experienced scientists. This version of MEGA is intended for the Windows platform, and it has been configured for effective use on Mac OS X and Linux desktops. It is available free of charge from http://www.megasoftware.net.

Introduction

The Molecular Evolutionary Genetics Analysis (MEGA) software was developed with the goal of providing a biologist centric, integrated suite of tools for statistical analyses of DNA and protein sequence data from an evolutionary standpoint. Over the years, it has grown to include tools for sequence alignment, phylogenetic tree reconstruction and visualization, testing an array of evolutionary hypotheses, estimating sequence divergences, web-based acquisition of sequence data, and expert systems to generate natural language descriptions of the analysis methods and data chosen by the user (Kumar et al. 1994, 2001; Kumar and Dudley 2007). With the fifth major release, the collection of analysis tools in MEGA has now broadened to include the maximum likelihood (ML) methods for molecular evolutionary analysis. Table 1 contains a summary of all statistical methods and models in MEGA5, with new features marked with an asterisk (*). In the following, we provide a brief description of methodological advancements, along with relevant research results, and technical enhancements in MEGA5.

Model Selection for Nucleotide and Amino Acid sequences

MEGA5 now contains facilities to evaluate the fit of major models of nucleotide and amino acid substitutions, which are frequently desired by researchers (Posada and Crandall 1998; Nei and Kumar 2000; Yang 2006) (fig. 1A). For nucleotide substitutions, the GTR and five nested models are available, whereas six models with and without empirical frequencies (+F) have been programmed for the amino acid substitutions (Table 1). MEGA5 provides the goodness-of-fit (see below) of the substitution models with and without assuming the existence of evolutionary rate variation among sites, which is modeled by a discrete Gamma distribution (+G) (Yang 1994) and/or an allowance for the presence of invariant sites (+I) (Fitch and Margoliash 1967; Fitch 1986; Shoemake and Fitch 1989). This results in an evaluation of 24 and 48 models for nucleotide and amino acid substitutions, respectively. For each of these models, MEGA5 provides the estimated values of shape parameter of the Gamma distribution (α), the proportion of invariant sites, and the substitution rates between bases or residues, as applicable. Depending on the model, the assumed or observed values of the base or amino acid frequencies used in the analysis are also provided. This information enables researchers to quickly examine the robustness of the estimates of evolutionary parameters under different models of substitutions and assumptions about the distribution of evolutionary rates among sites (fig. 1C). The goodness-of-fit of each model to the data is measured by the Bayesian information criterion (BIC, Schwarz 1978) and corrected...
Akaike information criterion (AICc, Hurvich and Tsai 1989) (see also Posada and Buckley 2004). By default, MEGAS lists models with decreasing BIC values (see below for the reason and caveats), along with log likelihood as well as AICc values for each model.

In the ML methods for evaluating the fit of substitution models to the data, an evolutionary tree is needed. MEGAS automatically infers the evolutionary tree by the Neighbor-Joining (NJ) algorithm that uses a matrix of pairwise distances estimated under the Jones–Thornton–Taylor (JTT) model for amino acid sequences or the Tamura and Nei (1993) model for nucleotide sequences (Saitou and Nei 1987; Jones et al. 1992; Tamura and Nei 1993; Tamura et al. 2004). Branch lengths and substitution rate parameters are then optimized for each model to fit the data. Users may provide their own tree topology in the Newick (New Hampshire) format for use in this model selection (fig. 1B). However, the automatic option is expected to be frequently used because trees are rarely known a priori. We tested the impact of the use of automatically generated trees in MEGAS on the process of model selection by computer simulation. These simulations used 448 sets of evolutionary parameters (base frequencies, sequence length, mean evolutionary rate, and transition–transversion rate ratio) derived from real sequence data (see Rosenberg and Kumar 2001) and introduced four different levels of rate variation among sites for each parameter set (Gamma shape parameter, \( \alpha = 0.25, 0.5, 1.0, \text{ and } 2.0 \)). Results showed that the best-fit models produced by using automatically generated trees were the same as those inferred using the true tree for \( \geq 93\% \) of the data sets according to the BIC and AICc criteria (fig. 2A).

For an overwhelming majority of data sets, AICc selected the most complex model (see also Ripplinger and Sullivan 2008). But, both BIC and AICc selected substitutions models that were more complex than the true model (Posada and Buckley 2004; Alfaro and Huelsenbeck 2006). The true model was among the top-3 when BIC was used and among the top-5 when AICc was used. When the rate variation among sites was extreme (\( \alpha = 0.25 \)), models incorporating invariant sites (\(+I\)) along with discrete gamma rate categories (\(+G\)) were favored for virtually every data set. This means that a discrete gamma (\(+G\)) model using a small number of categories (4), which is a common practice, coupled with an allowance for invariant sites (\(+I\)) is better at approximating the continuous Gamma distribution used in the simulation when the rate variation among sites is severe. This was confirmed by comparing the ML value for the fit of HKY + G model (10 categories) with the ML value for GTR + G + I model using only four discrete gamma categories. The former performed slightly better than the latter, even though the latter involved a more complex model.

On the basis of the above observation, we pooled \(+G\) and \(+G + I\) results for each model of substitution and found that BIC selected the true model for \( > 70\% \) of the data sets. In contrast, AICc selects the correct model only 35% of the time. Therefore, we rank the models by BIC in MEGAS (fig. 1C). However, the choice of criterion to select the best-fit models is rather complicated, and researchers should explore model selection based on AICc values and other available methods for evolutionary analyses in which choice of model is known to substantially affect the final result (e.g., Tamura et al. 2004; Ripplinger and Sullivan 2010). To facilitate downstream analysis to select the best model, MEGAS provides exporting of results in Microsoft Excel/Open Office and comma separated values formats.

These simulation results also provided us with an opportunity to evaluate the estimates of \( \alpha \) obtained by using the automatically generated tree and to compare them to those obtained by using the true tree under the correct model of substitution. The means and standard deviations of these estimates were very similar to the true values and virtually identical for automatically generated and true trees (fig. 2B). Similarly, the overall estimates of
transition–transversion ratio (R) were close to the true value for both automatically generated and true trees (fig. 2C). Therefore, the use of automatically generated trees with MEGA5 is useful, as a first approximation, in estimating evolutionary substitution parameters and evaluating relative fits of models.

**Inferring ML Trees**

MEGA5 now provides the ML method to infer evolutionary trees and conduct the bootstrap test for nucleotide and amino acid alignments (Felsenstein 1981, 1985). Because the ML method is computationally demanding, we provide heuristic methods that search for the ML tree by topological rearrangements of an initial tree (Swofford 1998; Nei and Kumar 2000; Guindon and Gascuel 2003; Stamatakis et al. 2005). The initial tree for the ML search can be supplied by the user (Newick format) or generated automatically by applying NJ and BIONJ algorithms to a matrix of pairwise distances estimated using a maximum composite likelihood approach for nucleotide sequences and a JTT model for amino acid sequences (Saitou and Nei 1987; Jones et al. 1992; Gascuel 1997; Tamura et al. 2004). For the user-selected data subset that contains sites with insertion–deletions and missing data, we begin by temporarily obtaining a site coverage parameter such that the number of ambiguous states and insertion–deletions per sequence are the lowest. This site coverage parameter is then used to generate data subsets for estimating evolutionary distances to build an initial tree along with branch lengths.
Comparison of the best-fit model identified by using MBE difference between the two sets of estimates was 0.2% (maximum 1.007 and 0.98, respectively. The absolute average difference simulated with a simulated 66-sequence data sets. (automatically generated and true trees for 1,792 computer 2734 F the gamma parameter (a) The relationship of true and estimated (B) The percentage of datasets for which the use of an automatically generated tree produces the same best-fit model as does the use of the true tree. Results are shown from datasets simulated with four different values of the gamma parameter (x) for rate variation among sites. (B) The estimates of x when using the automatically generated trees (filled bars) and the true tree (open bars). The average x and ±1 standard deviation are depicted on each bar; 10 discrete Gamma categories were used. (C) The relationship of true and estimated transition–transversion ratio, R, when using automatically generated trees for data simulated with x = 0.25. The value of R becomes 0.5 when the transition–transversion rate ratio, k, is 1.0 in Kimura’s two-parameter model. The slope of the linear regression was 1.005, with the intercept passing through the origin (r² = 0.98). Using the true tree, slope and r² values were 1.007 and 0.98, respectively. The absolute average difference between the two sets of estimates was 0.2% (maximum difference = 5.2%). Similar results were obtained for data simulated with x = 0.5, 1.0, and 2.0.

Fig. 2. Comparison of the best-fit model identified by using automatically generated and true trees for 1,792 computer simulated 66-sequence data sets. (A) The percentage of datasets for which the use of an automatically generated tree produces the same best-fit model as does the use of the true tree. Results are shown from datasets simulated with four different values of the gamma parameter (x) for rate variation among sites. (B) The estimates of x when using the automatically generated trees (filled bars) and the true tree (open bars). The average x and ±1 standard deviation are depicted on each bar; 10 discrete Gamma categories were used. (C) The relationship of true and estimated transition–transversion ratio, R, when using automatically generated trees for data simulated with x = 0.25. The value of R becomes 0.5 when the transition–transversion rate ratio, k, is 1.0 in Kimura’s two-parameter model. The slope of the linear regression was 1.005, with the intercept passing through the origin (r² = 0.98). Using the true tree, slope and r² values were 1.007 and 0.98, respectively. The absolute average difference between the two sets of estimates was 0.2% (maximum difference = 5.2%). Similar results were obtained for data simulated with x = 0.5, 1.0, and 2.0.

found this approach to produce better initial estimates when there are many insertions–deletions and missing data in the sequences. After this procedure, the user-selected data subset is restored and used in all subsequent calculations.

By default, MEGAS conducts an NNI search starting with the initial tree, such that the alternative trees differ in one branching pattern. One can expand the search space by using the CNI option in which alternative trees with two branches differences are evaluated (e.g., Nei and Kumar 2000, p. 126–127). In each case, ML values are computed for all the alternative trees produced by the branch swapping and all the branch swaps identified to increase the ML value are made simultaneously. If several single rearrangements are found to improve ML values for any branch, we choose the rearrangement that leads to the highest improvement in the ML value. We do not skip any branch swaps as long as it improves the ML value. In order to make major computational time savings, we do skip the evaluation of alternative topologies generated by rearrangements involving branches whose lengths are more than three times longer than their approximate standard errors. We use the second derivative of the ML score to generate approximate standard errors (Edwards 1972) during the branch length optimizations. Therefore, starting with systematic topological rearrangements of the initial tree, we discover trees with a higher ML value. These trees are subjected to new rounds of rearrangements, and this iterative process continues until no trees with greater likelihood can be found.

We tested the performance (time and accuracy) of the NNI and CNI searches in MEGAS by means of computer-simulated data sets containing 66 sequences (see Materials and Methods). We compared the time taken to complete these heuristic searches with each other and with those needed by PhyML version 3.0 (Guindon et al. 2010) and RaxML version 7.0 (Stamatakis 2006). Results showed that, on average, a CNI search requires twice the time of an NNI search in MEGAS (fig. 3A). Speeds of the MEGAS-NNI and MEGAS-CNI searches were similar to RaxML7-Mix and RaxML7-G, respectively. But, they were faster than PhyML3-NNI and PhyML3-SPR searches, respectively (fig. 3A). Similar trends were observed for another simulated dataset in which an increasingly larger number of sequences were analyzed (fig. 3B; 20–765 sequence data sets). For these data, the ML heuristic time increase shows a power trend with the increasing number of sequences (fig. 3B). It is important to note that the RaxML will be faster than MEGAS if the user’s machine is equipped with multiple processor and/or multicore CPUs because parallel versions of MEGAS are yet to be implemented.

Even though different programs and search options show large differences in computational times, the average accuracies of the inferred ML trees were found to be rather similar. The accuracy difference is less than 3% for the data sets containing 66 sequences (fig. 4A) and 765 sequences (fig. 4B). Therefore, ML methods in MEGAS appear to be comparable to other widely used ML implementations in terms of computational time and phylogenetic accuracy. In these simulations, we also compared the estimates of ML values generated by MEGAS and

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**Fig. 2.** Comparison of the best-fit model identified by using automatically generated and true trees for 1,792 computer simulated 66-sequence data sets. (A) The percentage of datasets for which the use of an automatically generated tree produces the same best-fit model as does the use of the true tree. Results are shown from datasets simulated with four different values of the gamma parameter (x) for rate variation among sites. (B) The estimates of x when using the automatically generated trees (filled bars) and the true tree (open bars). The average x and ±1 standard deviation are depicted on each bar; 10 discrete Gamma categories were used. (C) The relationship of true and estimated transition–transversion ratio, R, when using automatically generated trees for data simulated with x = 0.25. The value of R becomes 0.5 when the transition–transversion rate ratio, k, is 1.0 in Kimura’s two-parameter model. The slope of the linear regression was 1.005, with the intercept passing through the origin (r² = 0.98). Using the true tree, slope and r² values were 1.007 and 0.98, respectively. The absolute average difference between the two sets of estimates was 0.2% (maximum difference = 5.2%). Similar results were obtained for data simulated with x = 0.5, 1.0, and 2.0.
Inference of Ancestral States and Sequences

MEGA5 now provides inferences of ancestral states and sequences using the empirical Bayesian method (fig. 5). Given a phylogenetic tree, branch lengths are estimated under a user-selected model of nucleotide or amino acid substitution and the Bayesian posterior probabilities are generated for each possible ancestral state assignment for each node (Yang et al. 1995). With this addition, users can now explore ancestral sequences inferred using maximum parsimony and ML methods in MEGA5. However, the latter is often preferable because it helps investigators to distinguish among multiple equally likely (most parsimonious) assignments by using the posterior probabilities for each possible nucleotide or amino acid assignment. Furthermore, it is expected to produce more accurate results at positions that have undergone multiple substitutions over the whole tree or in specific lineages (Nei and Kumar 2000). These ancestral states, along with posterior probabilities, can be exported in multiple formats for individual positions and for all complete ancestral sequences. However, note that the reconstructed ancestral sequences are not real observed data and may involve systematic biases and random errors, especially for the highly variable positions, so caution should be exercised if they are to be used in further statistical analysis.

Position-by-Position Evolutionary Rates

For both nucleotide and amino acid sequence data, users can estimate relative rates of molecular evolution position-by-position in MEGA5. Users select the number of discrete categories to approximate the Gamma distribution, specify whether or not to model invariant positions, and choose a nucleotide or amino acid substitution model. As mentioned earlier, they can use an automatically generated
One can manually calibrate the molecular clock by setting vP trees using pairwise distances as well as the ML method. In the output, primary information along with the ages. With this addition, users can now produce linearized lengths by assuming equal evolutionary rate among lineages. However, such estimates should be used cautiously.

The divergence time for any one node in the tree, which produces divergence times for all other nodes in the tree. For these divergence times, MEGA5 calculates approximate confidence intervals from the variance of the node height computed using the curvature method (e.g., Schrag et al. 2006). Note that this procedure may underestimate the variance considerably due to the violation of the assumed clock constancy. The estimated node heights may be biased because of this reason, as well. So, the confidence intervals presented are not appropriate for hypothesis testing.

### Operating Systems and Platforms

We have also introduced many improvements to enhance MEGA’s usability. First, MEGA’s central user interface has now become activity driven where a launch bar provides direct access to the growing suite of tools according to the type of analysis needed through the “Action Bar” (fig. 6). Once a user selects what they wish to compute, MEGA5 prompts for a data file to use and the methods and data subsets to employ. This wizard-style layout will make MEGA easier for beginners and expert users alike. In this spirit, we have now added native support for the widely used FASTA file format for sequence data, and sequence data can now be aligned using the MUSCLE software, which is very fast and accurate for data sets containing a large number of sequences (Edgar 2004). Because MEGA now accepts user trees for heuristic searches, for molecular clock tests, and for ancestral sequence reconstruction, we have included a tree topology editor that is useful for creating trees and editing existing topologies by using drag-and-drop of branches.

### Usability Enhancements

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### Operating Systems and Platforms

In a recent survey of long-term MEGA users, we have found that both Mac OS X and Linux platforms are used by a substantial number of researchers (one out of four). Therefore, we have been optimizing the use of MEGAS on the Mac OS X and Linux platforms. For Mac OS X, we have now developed a custom installer that bundles MEGAS and the WINE software so that the installation of MEGAS is as simple as installing native Mac applications. WINE is a translation layer capable of running native Windows applications on POSIX compatible operating systems, such as Mac OS and Linux, and has two major advantages over using an emulation layer (i.e., virtualization software). First, by not using virtualization, users are not required to purchase a license for an additional operating system. Second, installation is simplified as there is no need to create and/or install an operating system disk image. As a result, Mac OS X users are able to use MEGAS as seamlessly as if they were operating it on the Windows platform for which MEGAS was originally developed. Similar provisions have been made for the use of MEGAS on the Linux platform. In our tests, we found that calculations in MEGAS on Mac OS X and Linux are <5% slower than Windows. This difference is rather small because all calculations via WINE are executed directly on the CPU like any other
native application in Mac OS X and Linux. In contrast, the MEGAS user interface is rendered via emulation by WINE, which can sometimes result in a slowdown when drawing on the screen. But, this is becoming less noticeable with contemporary CPUs that are extremely fast. This enhancement is likely to make MEGAS more useable for a greater number of researchers.

**Conclusion**

In summary, MEGAS now provides analysis tools for three major types (ML, MP, and evolutionary distances) of statistical methods of molecular evolution (table 1 and fig. 6). These facilities not only make MEGA useful for more researchers but also enable researchers to evaluate the robustness of their results by comparing inferences from multiple methods under a variety of statistical models. In the future, we will continue to develop MEGA with a focus on implementing faster algorithms for phylogenetic inference, integrating more third party tools, and upgrading the computing core to use multicore and distributed computing effectively. As always, all versions of MEGA are available free of charge from http://www.megasoftware.net.

**Materials and Methods**

We generated two sets of nucleotide sequence data by using computer simulations. In one, a 66-taxa tree representing the phylogenetic relationships among mammals was used (see fig. 1 in Rosenberg and Kumar 2001). We simulated DNA evolution for 448 hypothetical genes along this tree, each with an independent set of evolutionary parameter values (base frequencies, sequence length, mean evolutionary rate, and transition–transversion rate ratio) estimated from the real sequence data (Rosenberg and Kumar 2003). For each set of evolutionary parameters (448 different sets), the branch lengths of the model tree were estimated using the corresponding evolutionary rate. Sequence alignments were generated under the Hasegawa–Kishino–Yano (HKY) (Hasegawa et al. 1985) model of nucleotide substitution at four different levels of rate variation among sites ($x = 0.25, 0.5, 1.0, and 2.0$) that were implemented during computer simulations via a discretized gamma distribution with a very large number of categories. This resulted in a total of 1,792 alignment sets.

We also generated DNA sequence alignments containing 20–765 taxa, which were based on the corresponding sized trees derived from a master phylogeny of 765 taxa (see supplementary fig. S1, Supplementary Material online, in Battistuzzi et al. [2011]). This master phylogeny was obtained by pruning taxa and groups from the tree of 1,671 families in the Timetree of Life (Hedges and Kumar 2009), such that the final tree was strictly bifurcating. The resultant tree of 765 taxa was scaled to time and spanned 4.2 billion years of evolution. This master topology was subsampled to produce model trees used to generate the sequence alignments containing varying number of taxa (20, 40, 60, ..., 500), with one set containing all 765 taxa. Sequences were simulated using SeqGen (Rambaut and Grassly 1997) under the HKY (Hasegawa et al. 1985) model of nucleotide substitution with a $G + C$ content of 48% and a transition–transversion rate ratio of 1.05, which were estimated from an alignment of small subunit rRNA sequences from 800 taxa of animals, fungi, plants, and archaeabacteria. In order to make the evolutionary rate heterogeneous among tip and internal lineages, rates were varied randomly by drawing them from a uniform distribution with boundaries $\pm 5\%$ of the expected rate in each branch independently. We used substitution rates of $0.025, 0.050, and 0.100$ per base pair per billion years to establish branch lengths. In total, 530 data sets were generated in this way and the results are presented in the main text. We also conducted 290–765 taxa simulations in which sequences evolved four times faster (0.4 substitutions per site per billion years) and found the differences between methods were very similar to those reported here (results not shown).

A benchmark comparison of ML phylogenetic inference between MEGAS, RAxML7, and PhyML3 was performed for all simulated datasets by collecting the computational and phylogenetic performance of these programs, including
execution time (in seconds), the estimate of a gamma shape parameter, ML values, and topological accuracy. Because Windows is MEGA5’s native operating system, Windows executables were used for PhyML (version 3.0) and RAxML (version 7.04). All analyses were conducted on computers with identical hardware (Intel Q8400 2.66 GHz Quad Core processor and 6 GB RAM) and operating systems (64-bit Windows 7 Enterprise Edition). For direct comparison, each program was executed serially in a single thread of execution with one core utilized per dataset.

In order to generate comparable results on time and accuracy, we used identical substitution models and discrete gamma options across all programs. Because the fastest heuristic search in RAxML, MIX, assumes a general time reversible (GTR) model with four discrete gamma rate categories, we used these options in all cases, unless noted otherwise. For all three programs, analyses were conducted using the automatically generated initial trees and selecting the default options. And, heuristic searches starting with the initial trees were conducted with two different levels of branch rearrangements: quick searches (Nearest Neighbor Interchange [NNI] for MEGA5 and PhyML and MIX for RAxML) and slow searches (Close Neighbor Interchange [CNI] for MEGA5, Subtree–Pruning–Regrafting [SPR] for PhyML, and GTRGamma for RAxML). The accuracy of phylogenetic tree of $\phi$ was estimated from the topological distance ($d_T$) between the inferred tree and the true topology was given by $(n - 3 - \frac{1}{2}d_T)/(n - 3)$.

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