Experimental Phylogeny of Neutrally Evolving DNA Sequences Generated by a Bifurcate Series of Nested Polymerase Chain Reactions

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A known phylogeny was generated using a four-step serial bifurcate PCR method. The ancestor sequence (SSU rDNA) evolved in vitro for 280 nested PCR cycles, and the resulting 15 ancestor and 16 terminal sequences (2,238 bp each) were determined. Parsimony, distance, and maximum likelihood analysis of the terminal sequences reconstructed the topology of the real phylogeny and branch lengths accurately. Divergence dates and ancestor sequences were estimated with very small error, particularly at the base of the phylogeny, mostly due to insertion and deletion changes. The substitution patterns along the known phylogeny are not described by reversible models, and accordingly, the probability substitution matrix, based on the observed substitutions from ancestor to terminal nodes along the known phylogeny, was calculated. This approach is an extension of previous studies using bacteriophage serial propagation, because here mutations were allowed to occur neutrally rather than by addition of a mutagenic agent, which produced biased mutational changes. These results provide for the first time biochemical experimental support for phylogenies, divergence date estimates, and an irreversible substitution model based on neutrally evolving DNA sequences. The substitution preferences observed here (A to G and T to C) are consistent with the high G+C content of the Thermus aquaticus genome. This suggests, at least in part, that the method here described, which explores the high Taq DNA polymerase error rate, simulates the evolution of a DNA segment in a thermophilic organism. These organisms include the bacterial rod T. aquaticus and several Archaea, and thus, the method and data set described here may well contribute new insights about the genome evolution of these organisms.

Introduction

Experimental phylogenetics is a convincing means of understanding basic processes of nucleotide change in phylogenies (Hillis, Mable, and Moritz 1996). Hillis and collaborators evolved the bacteriophage T7 by sequential propagation and generated a known phylogeny which provided for the first time experimental support for phylogeny inference methods (Hillis et al. 1992; Bull et al. 1993). Gene phylogenies can be inferred from sequence data using several algorithms under three basic optimality criteria, namely, parsimony (Fitch 1977), pairwise distances (Saitou and Nei 1987), and maximum likelihood (Felsenstein 1981). Among these, the use of explicit models of sequence evolution, or maximum likelihood, which is computationally very intensive, has been increasing recently because of improvements in computer hardware speed and software optimization (Olsen et al. 1994; Strimmer and von Haeseler 1996; Korber et al. 2000). Gene phylogenies may represent species phylogeny if the substitutions in a particular gene represent orthologous steps (Li and Graur 1991). Divergence dates of genes and species can also be estimated from phylogenetic distances (Rambaut and Bromham 1998; Yoder and Yang 2000). These estimates are based on the concept of a molecular clock (Zuckerkandl and Pauling 1962), either global or local, which can be tested for a set of sequences, models, and trees, using relative rate tests (Sarich and Wilson 1973), trip-
mostly reflect the selection regime and thus may cause convergent substitutions, under negative selection, or anomalously long phylogeny branches, under positive selection. Accordingly, divergence dates could be underestimated or overestimated, respectively.

Here we present a method, based on serial PCR, that extends previous studies (Hillis et al. 1992; Bull et al. 1993) by generation of a data set of neutrally evolving sequences. Analysis of this data set provided experimental support for maximum likelihood, with model fit analysis, for finding the correct topology, reconstruction of ancestor sequences, and divergence date estimates. This data set was also used to calculate a probability transition matrix which describes an irreversible dynamics, based on Taq DNA polymerase error rate, and should contribute to further research on coalescence theory (Kingman 1982), phylogeny inference methods, and evolution of thermophilic organisms (Galtier, Tourasse, and Gouy 1999).

Materials and Methods
Gene Amplification and Sequencing

The ancestor sequence (Trypanosoma cruzi SSU rDNA, GenBank AF288660) was used as template in a 35 cycle PCR using primers RIBA (5'-CCGA-ATTGCTCGACAACCTGGTTGATCCCTGCAA GT-3') and RIBB (5'-CCGGGATCCAAGCTTGATCCCTTC TGCAAGTTTCCACTAC-3') which enables the amplification of the complete SSU rDNA sequence. The amplification started with amplification of 0.35 μg of cloned ancestor sequence (0.102 pmol, 6.14 × 10^10 molecules) in a 100-μl solution containing 5 units of Taq DNA pol (GIBCO), 7 mM MgCl₂, 40 nmol each deoxyribonucleotide triphosphates (dNTP), 20 mM (hydroxyethyl)aminomethane (Tris)–HCl pH 8.4, 50 mM KCl, and 10 pmol of each primer. After 35 cycles amplicons were purified from gel (GFX, Amersham-Pharmacia) serially diluted 1:1,000, and 4 μl of the diluted solution were used as template for an additional 35 cycles in 100-μl solutions containing 5 units of Taq DNA pol (GIBCO), 7 mM MgCl₂, 40 nmol each dNTP, 20 mM Tris–HCl pH 8.4, 50 mM KCl, and 10 pmol of each primer (fig. 1). Cycling conditions were 94°C for 1 min, 41°C for 1 min, and 72°C for 2 min, and final extension 72°C for 7 min. After each 70 nested cycles the amplicons were cloned into pBluescript, and two randomly selected clones were picked to be the ancestor of another 70 cycles nested for a total of four rounds of 70 nested cycles. This procedure was repeated four times, resulting in four rounds of 70 nested PCR cycles. Amplification of cloned products was done using M13 forward and reverse primers. The nested reaction has 280 cycles, and all 31 sequences derived from the process (16 terminal nodes plus 15 ancestors), along with six additional clones of the first 70 cycles, were determined completely, in a total of 37 complete SSU rDNA sequences (total of 82,806 assembled bases). Sequencing was done using BigDye Terminators (Applied Biosystems) in an ABI377/96 auto-

FIG. 1.—Evolution of DNA sequences by a series of bifurcate PCRs. An ancestor SSU rDNA cloned in pBluescript was used as template for series 1 of 70 nested PCR cycles with M13 primers. After the initial 35 cycles, reaction products were diluted 1:1,000 and used as templates for the subsequent 35 cycles, with rDNA primers RIBA and RIBB. After 70 cycles amplicons were cloned, and two clones were picked randomly and used as templates for the next series of nested PCR cycles. Lineages are propagated at random, and therefore the evolution is neutral and behaves as a stochastic process. Tree nodes T1 to T16 indicate terminal sequences, and 1.1 to 4.8, internal ancestors.
Fig. 2.—Polymorphic sites of sequences generated by serial PCR neutral evolution. Sequences 1.1 to 4.8 represent the internal ancestors, and T1 to T16, the terminal sequences. Sequences 2.3 to 2.8 were not used in subsequent propagation, except to estimate the Taq DNA polymerase error rate at 70 cycles. Numbers above sequences indicate the position number in the alignment (total number of positions considered is 2,238). Dots indicate residues that are identical to sequence 1.1.

imated sequencer, by primer walking using internal primers of the 18S rRNA gene. Sequences were assembled using PHRED+PHRAP+CONSED (Gordon, Abajian, and Green 1998) from ABI chromatograms, available upon request. Quality of assembled sequences ranged from phred scores 30 to 40 as estimated by CONSED (Gordon, Abajian, and Green 1998). Sequences presented here have been deposited in GenBank under accession numbers AF288660 (ancestor 1.1) and AF359461 to AF359496 (from 2.1 to T16). Supplementary data, such as alignments and phylogenies, are available on the World Wide Web site (http://compbio.epm.br/ievol) of one of us.

Phylogenetic Analysis

Terminal sequences were aligned by eye using SEAVIEW sequence editor (Galtier, Gouy, and Gautier 1996). Trees were constructed using PAUP 4.0b6 (Swofford 1998) and TREE-PUZZLE (Strimmer and von Haeseler 1996), and modelfit tests were performed using the hierarchical likelihood ratio test implemented in MODELTEST 3.04 (Posada and Crandall 1998). Maximum likelihood trees were constructed using the model selected by MODELTEST (TVMef [Posada and Crandall 1998] with equal base frequencies; the rate matrix A–C = 0.4397, A–G = 13.2362, A–T = 4.9778, C–G = 0.2123, C–T = 13.2362, and G–T = 1.0; proportion of invariant sites = 0; and equal rates for all sites) with heuristic tree-bisection–reconnection (TBR) search. Parsimony (Fitch 1977) trees were built using accelerated transformation (ACCTRAN) and TBR searching with collapse option. Distance trees were constructed using Neighbor-Joining (Saitou and Nei 1987) with a maximum likelihood distance matrix using the model selected by MODELTEST. The standard errors of branch lengths were estimated using PAUP 4.0b6 (Swofford 1998). The number of molecules after 280 cycles was calculated by quantitation of templates and PCR products by absorbance at 260 nm, applying corresponding corrections for dilutions, and conversion to number of molecules using Avogadro’s number. Divergence dates with low and high confidence intervals and associated evolutionary rates were estimated using the quartet analysis implemented in QDATE version 1.11 (Bromham et al. 1998; Rambaut and Bromham 1998). The number of substitutions of each rate category was directly quantitated from polymorphic sites along the real phylogeny.

Model Construction

The instantaneous rate matrix (Q-matrix) was built from the observed number of changes of each of 16 categories, in the real topology. Each element Qij was calculated by dividing the observed number of changes from nucleotide i to nucleotide j by the total number of changes. The diagonal elements Qii were calculated using the relation Qii = −Σj≠i Qij. The Q-matrix was then written as in table 1.

The substitution probability matrix (P-matrix) was calculated from the relation Pt(t) = eQt (Swofford et al. 1996). In order to find the elements of the P-matrix we had to decompose the Q-matrix into its eigenvalues and eigenvectors. The eigenvalues were obtained by calculating the λ values (vα) that satisfied the equation det(Q − λI) = 0 (where I is the identity matrix), namely, v1 = −0.5187, v2 = −0.4218, v3 = −0.0595, and v4 = −5.7958 × 10−6. For each λ value the respective eigenvector (VA) was determined as that satisfying the equation (Q − λI)Vλ = 0. These vectors are: V1 = (−0.6060, −0.1701, 0.1669, 0.7589); V2 = (0.5025, −0.2510, −0.2801, 0.7785); V3 = (0.5243, −0.5155, 0.6207, −0.2722); and V4 = (−0.4999, −0.5001, −0.4998, −0.5000). The P(t) elements in the P-matrix were calculated using standard transformation procedures of the Q-matrix, using the previously calculated eigenvalues and eigenvectors. All mathematical calculations were confirmed using MATHEMATICA software (Wolfram Research).

Results and Discussion

Here we wanted to simulate neutral evolution and therefore we present a model which explores the high error rate of Taq DNA polymerase (Saiki et al. 1988) (fig. 1). A known SSU rRNA gene sequence was used as the ancestor to start the process of sequential PCR
evolution (fig. 1). Every 70 cycles the PCR products were cloned, and two clones were completely sequenced and used as templates (ancestors) for the next round of 70 cycles (fig. 1). Accordingly, the ancestor 1.1 originated 16 terminal sequences or terminal nodes, T1 to T16, after 280 PCR nested cycles (generations) (fig. 1). The full-length sequences of the 16 terminal nodes and the 15 internal nodes (2.1 to 4.8, fig. 3A), or ancestors, were determined. The alignment of all 37 sequences used here revealed 196 polymorphic sites, including gaps, and the alignment of the 16 terminal sequences had 169 polymorphic sites (fig. 2). After PCR evolution of 280 generations (fig. 1), the total number of molecules, \(9.92 \times 10^{11}\), was estimated from PCR product quantitation. Sequences 2.3 to 2.8 were used only to calculate the mutation rate of Taq DNA polymerase after 70 PCR cycles, as described later.

The real phylogeny obtained (fig. 3A) has a series of 15 dichotomies from the initial ancestor to the 16 terminal sequences, and substitutions that occurred along the phylogeny involved 7.6% of the total number of positions (table 1). We observed that the number of substitutions per time interval (along a branch length) varies significantly. This might be due to stochastic effects once lineages are sampled and propagated randomly. The 16 terminal sequences (T1 to T16) were used to reconstruct the real phylogeny by maximum likelihood, parsimony, and neighbor-joining (Fitch 1977; Felsenstein 1981; Saitou and Nei 1987). Topologies obtained by the three methods were identical and found the real topology. In fig. 3B we show the reconstructed maximum likelihood phylogeny which has a topology identical to the true tree (fig. 3A). This phylogeny has ln(Likelihood) = −4259.7384 and was inferred using a
The serial PCR in vitro evolution resulted in the topology depicted (A) with varying branch lengths whose ancestors (1.1 to 4.8, circled) and terminal sequences (T1 to T16) were sequenced in full length. Scale bar indicates the number of cycles between tree internodes and nodes. The inferred phylogeny (B) has a topology identical to the real tree (A) and 9 out of 30 branch lengths were estimated correctly. Boxed numbers indicate branch lengths (number of substitutions), numbers in italics represent the percentage of a given cluster in 100 bootstrap replicates, with reestimation of parameters at each bootstrap replicate (top), and without reestimation at each replicate (bottom). Numbers below arrows indicate the estimated divergence (cycles ago), with the low–high confidence interval range (in parenthesis) as calculated by maximum likelihood quartet analysis (Rambaut and Bromham 1998). Numbers of substitutions, with corresponding standard errors, in the inferred tree (B) were calculated by multiplying the branch lengths (in substitutions per site) by the total number of positions (2,238 bp).

We also tested if divergence times could be estimated from inferred phylogenies. The 16 terminal sequences were analyzed by a maximum likelihood quartet method (Rambaut and Bromham 1998), using the same substitution model and parameters used to infer the maximum likelihood phylogeny (fig. 3B). All terminal pairs diverged 70 cycles earlier and were clustered into quartets to infer the divergence of internal nodes. Divergence times of ancestors 1.1, 2.1, 2.2, 3.2, and 3.4 were estimated correctly, and ancestors 3.1 and 3.3 had dates outside the 95% confidence interval. The inferred tree, however, passes the likelihood ratio test for molecular clocks (Felsenstein 1988) as the difference between the likelihoods of the molecular clock constrained tree and the unconstrained tree is not statistically significant at 95% confidence level (2 × ΔLnL = 21.2818, and the
Chi-square critical value for 14 degrees of freedom at P < 0.05 is 23.685). The maximum likelihood quartet analysis also estimated the evolutionary rate between $0.24 \times 10^{-3}$ and $0.42 \times 10^{-3}$ substitutions per site per generation.

Ancestor sequences 1.1 to 4.8 were also predicted from the maximum likelihood tree (hypothetical ancestors, HA) and compared with ancestors from the real phylogeny (fig. 4). Ancestors of the real phylogeny were reconstructed with accuracy from 99.46% to 99.87% (3 to 12 differences) by maximum likelihood. Most of the inaccurately assigned ancestor states were in regions with insertions and deletions and in two positions with two substitutions at the same site, positions 101 and 637 (fig. 4 and table 1). The reconstruction with the most errors (12) was ancestor 1.1, and the most accurate reconstructions were from ancestors 3.4, 4.7, and 4.8 (fig. 4). This suggests that as we move deeper in the tree, ancestor sequence reconstructions might be more sensitive to insertion and deletion events and multiple substitutions at the same site. Insertions and deletions occurred much more frequently after runs of homopolymeric regions, as observed elsewhere, and might be caused by the slippage of Taq DNA polymerase (Bracho, Moya, and Barrio 1998).

The substitution model selected by the hierarchical likelihood ratio test was compared with the actual changes (table 2). It shows that the changes in the real tree follow an irreversible model, particularly when A->G-G, A->A-T-T, and C->T-T changes are compared. Base frequencies in the data set (16 terminal sequences) are f(A) = 0.24938; f(C) = 0.22548; f(G) = 0.26574; and f(T) = 0.25941; among constant sites they are (0.24374, 0.23225, 0.27171, 0.25230) and among variable sites (0.32895, 0.12972, 0.18143, 0.35990). This suggests that the direction of change is not biased toward more abundant residues in the nucleotide pool.

To depict the probability of substitutions along the real phylogeny, the elements of the instantaneous rate matrix, the Q-matrix (table 2), were used to assemble the following equations:

\[
\frac{dP(A)}{dt} = -0.3595P(A) + 0.0065P(C) + 0.1242P(C) - 0.0065P(G) - 0.0915P(T)
\]

\[
\frac{dP(C)}{dt} = 0.0065P(A) - 0.1046P(C) + 0.0000P(G) + 0.2810P(T)
\]

\[
\frac{dP(G)}{dt} = 13.2362P(A) + 0.0000P(C) - 0.1503P(G) + 0.0131P(T)
\]

\[
\frac{dP(T)}{dt} = 4.9778P(A) + 0.0980P(C) - 0.3856P(T)
\]
Table 3
Substitution Probability Matrix Elements of the \( P \)-matrix, Derived from Observed Substitutions Along the Real Phylogeny (fig. 3A)

<table>
<thead>
<tr>
<th>( P_{ij}(t) )</th>
<th>( j = A )</th>
<th>( j = C )</th>
<th>( j = G )</th>
<th>( j = T )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>i = A</strong></td>
<td>( 0.4361e^{-0.5187} + 0.2991e^{-0.5187} )</td>
<td>(-0.1199e^{-0.5187} + 0.1449 )</td>
<td>(-0.721e^{-0.5187} + 0.2296e^{-0.5187} )</td>
<td>(-0.3288e^{-0.5187} + 0.2959e^{-0.5187} )</td>
</tr>
<tr>
<td><strong>i = C</strong></td>
<td>(-0.0159e^{-0.5187} + 0.1449 )</td>
<td>(-0.4108e^{-0.5187} + 0.1492 )</td>
<td>(-0.3683e^{-0.5187} + 0.2898 )</td>
<td>(-0.0776e^{-0.5187} + 0.1459 )</td>
</tr>
<tr>
<td><strong>i = G</strong></td>
<td>(-0.1201e^{-0.5187} - 0.1667e^{-0.5187} )</td>
<td>(-0.4039e^{-0.5187} + 0.1493 )</td>
<td>(-0.3622e^{-0.5187} + 0.2899 )</td>
<td>(-0.0922e^{-0.5187} - 0.1298e^{-0.5187} )</td>
</tr>
<tr>
<td><strong>i = T</strong></td>
<td>(-0.5461e^{-0.5187} + 0.1449 )</td>
<td>(-0.4863e^{-0.5187} + 0.1493 )</td>
<td>(-0.4360e^{-0.5187} + 0.2899 )</td>
<td>(-0.0915e^{-0.5187} + 0.1459 )</td>
</tr>
</tbody>
</table>

**Note.**—Given \( \alpha = 0.5187 \), \( \beta = 0.4218 \), and \( \gamma = 0.0395 \).

The results presented here, using a biochemical approach, are consistent with the likelihood topology reconstruction method and the maximum likelihood model selected. The experimental approach to phylogeny inference, which involves synthesizing RNA from the known phylogeny (fig. 3A), allows for the modeling of substitution events, including those driven by errors of an enzymatic reaction. The process of substitution is driven by errors in the enzymatic reaction, which is further verified by the results of computer simulations. Consequently, the results obtained under this process will depend on the particular parameters and bias that the programmer chooses to include.
ture, and most substitutions tend to occur within regions of unpaired loops (Hillis and Dixon 1991).

The serial PCR method described here could be used in studies of evolution of thermophilic organisms. The substitution bias observed here (A > G and T > C, table 1) is consistent with the high G + C content of the Thermus aquaticus genome, and might be a consequence of specific properties of Taq DNA polymerase. However, the polymerase domains of Taq DNA polymerase and the Klenow fragment of Escherichia coli DNA polymerase I are nearly identical. In Taq DNA polymerase, two of the catalytically critical carboxylate residues on the 3'-5' exonuclease activity are missing (Kim et al. 1995). The simulation described here might well reflect the neutral evolution of a DNA segment in a thermophilic organism, such as in the bacterial rod T. aquaticus and in several Archaea, which implies that the method presented here could be developed to address questions about the genome evolution of these organisms (Woese 1987; Pace 1997).

As a perspective, the serial PCR evolution method and the data set presented here can contribute to future studies on coalescence theory (Kingman 1982) and to divergence date estimate methodology (Rambaut and Bromham 1998; Yoder and Yang 2000), because the number of generations, the phylogeny, and the mutation rate per generation are known. The in vitro generation of a phylogeny with no selection and no migration should be particularly useful for estimating the θ parameter (Kuhner, Yamato, and Felsenstein 1995). Nevertheless, this study provides for the first time biochemical experimental support for phylogeny inference from neutral substitutions using maximum likelihood with model optimization.

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