# SIMULATIONS OF EVOLUTIONARY RADIATIONS AND THEIR APPLICATION TO UNDERSTANDING THE PROBABILITY OF A CAMBRIAN EXPLOSION

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ABSTRACT—A molecular survey of animal phylogeny (Wray et al., 1996) recovered the presumed correct temporal order of the phylogenetic splits Protostomata-Deuterostomata, Echinodermata-Chordata, and Agnatha-Gnathostomata in studies of six of seven gene sequences. This result raised the question of how this order could be recovered if all of the phyla had appeared in a Cambrian "explosion" of less than 10 m.y., given the expected erratic nature of the molecular "clock." We simulated trees, and molecular sequence evolution along the trees, under different evolutionary radiation scenarios, with different periods of radiation, relative to times of subsequent evolution. Simulations and phylogenetic analyses of sequences derived from a simulated "Cambrian explosion" of 10– 35 million years did not allow the successful recovery of the correct tree, using neighbor-joining, maximum likelihood, or parsimony methods. Success in recovering phylogenies under a Cambrian divergence scenario (520 million years ago) did not exceed 80 percent without an extended divergence time interval of at least 100 m.y. An increased substitution rate during the initial radiation improved the ability to recover correct phylogenies, especially when the rate was 8–10 times the rate following the radiation. Our results militate against the likelihood of an Early Cambrian or slightly longer explosion of the animal phyla, as apparently supported by the fossil record. Some limitations to these conclusions are discussed.

## INTRODUCTION

WRAY ET AL. (1996) used molecular data to test the Cambrian explosion hypothesis and concluded the divergence of the protostomes and deuterostomes occurred much earlier than the Cambrian, perhaps as deep as 1.2 billion years ago. The echinoderm-chordate split was estimated at approximately one billion years and was separated from the protostome-deuterostome divergence by an interval greater than 100 m.y. Thus the divergence of animal phyla was neither Cambrian nor explosive. Subsequent molecular estimates of the Protostome–Deuterostome split have all yielded Precambrian ages; all but two of 13 cited in Levinton (2001) were deeper than 800 m.y.a. (see for example: Ayala et al., 1998; Nikoh, 1997; Bromham et al., 1998; Gu, 1998; Wang, 1998; Hausdorf, 2000; Levinton, 2001). The ages from these estimates, despite being all Precambrian, cover a discouragingly broad range, which is a probable reflection of differences of methodology, different genes employed, and likely real variation in rates and variance of rates of evolution along different branches of the evolutionary tree.

We can calculate from the data in Wray et al. (1996) a probability of getting the correct order of appearances of the protostome-deuterostome, echinoderm-chordate, and agnathan-gnathostome evolutionary splits from the seven sequences (Table 1). For example, there are six permutations of order of appearance for the three evolutionary splits mentioned above. Therefore any analysis will get the correct order by chance one out of six times (for some of the genes, we only have two splits, which yield only two permutations). If the genes are independent, the probability of getting the order of appearance of splits correctly is the product of the individual probabilities. Such assumption of independence of estimates among genes yields a probability of 0.00016 that the order of splits we got were achieved by chance alone. If rates of the mitochondrial genes are correlated owing to linkage, but the nuclear genes are independent of each other and independent of the mitochondrial genes, then the probability of obtaining the order of evolutionary splits found for the different divergences is 0.0348. This latter assumption is overly conservative, since great spans of time would tend to reveal differences related more to differences in substitution rate among the genes, which are considerable.

Given the vagaries of the molecular clock, we would not expect to get a correct *order* of appearance in calculations from such different genes if the animal phyla diverged in just a few million years, as is suggested by the Early Cambrian fossil record, calibrated by radioisotopic estimates (Bowring et al., 1993). We now have simulated both evolutionary trees and nucleotide sequences and find that it is unlikely that *any* correct resolution of a tree would be possible under a strict Cambrian explosion scenario. Indeed our evidence suggests that the divergence of the animal phyla likely occurred over a protracted period, possibly greater than 100 million years, which fits previous molecular results (Wray et al., 1996).

There are three separable conceptions of the extent of the period of the diversification of the animal phyla. The extreme Cambrian explosion hypothesis (Gould, 1995, p. 681) argues that the divergence of the animal phyla ". . . lasted only 10 million years (from 530–520 million years ago) and featured the first appearance in the fossil record of effectively all modern animal phyla, including annelid worms and chordates." This interpretation is based upon a literal interpretation of a large number of animal phyla in the Precambrian, which corresponds to a time interval of <10 million years (Bowring et al., 1993). The late Precambrian divergence hypothesis, in contrast, restricts the divergence of the animal phyla to the latest Vendian and Early Cambrian, which encompasses a rapid appearance of trace fossils and identifiable triploblastic metazoans (Valentine et al., 1996). This hypothesis restricts the protostome-deuterostome branching and diversification of derived triploblastic metazoan clades to a period of about 35 m.y. (565–530 m.y.a.). Finally, the deep Precambrian hypothesis argues that the divergences predate the Vendian, and that the divergences of the triploblastic metazoan clades may have extended over a significant period of time, perhaps over 100 m.y. (Wray et al., 1996; Fortey et al., 1996).

The timing and temporal extent of the radiation of the Metazoa must affect our ability to resolve evolutionary relationships of the animal phyla with molecular data (Philippe et al., 1994). With increasing age of the divergence, multiple hits at specific nucleotide or amino acid sites are more likely, which obscures the informative sequence substitutions needed to resolve relationships. Furthermore, as a series of divergences are confined to shorter and shorter periods of time, it stands to reason that the informative amount of evolutionary change will be small, relative to subsequent evolution of the divergent lines. Thus an ancient

TABLE *1*—Rank order of divergence times for estimates of animal phyla divergences in Wray et al. (1996), and probability of getting expected divergence order. With three divergence times (e.g., ATPase 6) there are six permutations whereas with two estimates (e.g., Hemoglobin) there are just two permutations. nd = no data.

| | Gene<br>Gene type | ATPase 6<br>Mitoch. | Cyt. C<br>Nuclear | COI<br>Mitoch. | COII<br>Mitoch. | Hemoglobin<br>Nuclear | NADH<br>Mitoch. | 18S rRNA<br>Nuclear |
|---|---|---|---|---|---|---|---|---|
| Protostome-Deuterostome | | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Echinoderm-Chordate | | 2 | 1 | 2 | 2 | 2 | 2 | 2 |
| Agnathan-Gnathostomata | | 1 | 2 | 1 | 1 | nd | 1 | nd |
| Probability | | 0.167 | 0.833 | 0.167 | 0.167 | 0.5 | 0.167 | 0.5 |

but short-lived evolutionary radiation will be difficult to resolve with standard tree-building techniques. Uneven rates of evolution along different branches may further reduce our ability to resolve trees (Felsenstein, 1988; Huelsenbeck and Hillis, 1993).

### METHODS

Our simulations (Fig. 1) take the following form: An initial ancestral lineage, bearing an ancestral DNA sequence of $x$ nucleotides, splits into $n$ lineages over a divergence interval of time $d$; branching occurs via a pure birth process (Kuhner and Felsenstein, 1994). This means that taxa arise but do not become extinct. This type of model is to be distinguished from a birth-death model, where taxa also become extinct according to a specified protocol. We chose a birth-only model because of our method of analyzing tree-building success, which involved creating a specific number of taxa (see below). Extinction eliminates taxa and, at high levels, can degrade the ability to calculate an accurate tree from the characters in the residual group of surviving taxa (see discussion and references in Rosenberg and Kumar, 2001). Thus a birth-only model should give a best-case scenario on the success of recovering accurate trees following an evolutionary radiation.

The distribution of nodes is random in this branching model, and therefore there are few, if any, trees that have discordant branch lengths, placing the trees in either the "Felsenstein Zone" (Huelsenbeck and Hillis, 1993) or the "Farris Zone" (Siddall, 1998). The length of these branches is normalized such that the total divergence time totals to $d$. The $n$ taxa continue to evolve at the same rate of sequence change for a period $c$ without further splitting. We can imagine a Cambrian explosion occurring over a



FIGURE *1*—Setup of simulation model for 4 taxa.

period of $d = 10$ m.y., with subsequent molecular evolution continuing for $c = 520$ m.y. The evolution of 1,000 nucleotides of DNA was simulated along the tree for most of our work, although we also investigated the effect of number of nucleotides on tree recovery. Molecular evolution was simulated according to the Kimura two-parameter model, using a transition to transversion ratio of 2. We have simulated a set of $n$ sequences, corresponding to $n$ terminal taxa that were produced over varying values of $d$, ranging from a Cambrian explosion of $d = 10$ m.y. to a protracted period of $d = 500$ m.y. One can see this problem as whether or not one can calculate correct phylogenies under different values of c/d. As this value increases, the radiation period becomes more and more brief, relative to the time of subsequent occurrence of the taxon, and correct phylogenies should be more and more difficult to recover.

We performed a preliminary study to bracket the rate of nucleotide substitution that maximized accuracy of tree recovery. A rate that is too slow will result in very little evolution at each nucleotide site, which provides very little change and, therefore, provides little information or phylogenetic signal. On the other hand, a rate that is too fast erases the phylogenetic signal owing to subsequent multiple changes at any nucleotide site. We used this optimal intermediate rate to produce a "best-case" scenario of tree recovery under a Cambrian explosion. The rate that produced the most accurate trees for a Lower Cambrian divergence time (c = 520) was 0.0001, although rates of substitution half of an order of magnitude faster or slower did not give substantially different results. Importantly, the rate in this model did not change from the divergence period to the postdivergence period, i.e., it conformed to a molecular clock hypothesis. We relax this assumption below.

Sequences were simulated by using homogeneous rates for all sites, and by simulating variability of rates among sites by means of a discontinuous categorization of a gamma distribution (Yang, 1993a), using 10 categories and with the shape parameter $\alpha = 0.5$ (Yang, 1993b). This distribution is meant to approximate the variation in substitution rate that has been found among sites in studies of molecular evolution (Yang, 1993b). Most simulations were run with four terminal taxa, which can be grouped into only three alternative unrooted topologies. Thus under random circumstances, one will obtain the correct tree one-third of the time. This provides a convenient baseline random expectation for an estimate of the efficacy of a given tree-building method to obtain the correct tree (Hillis et al., 1994; Huelsenbeck and Hillis, 1993). Our runs were consistent with the expectation that increasingly poor performance would converge upon a frequency of correct trees of one-third (see results).

We used three different methods (Felsenstein, 1993) to construct trees from the simulated nucleotide sequences: Neighbor-joining (Saitou and Nei, 1987); maximum-likelihood (Felsenstein, 1981); and parsimony (Farris, 1970). We used *Phylip* (Felsenstein, 1993) to calculate trees for all three techniques. In the case of neighbor joining, a single shortest tree is recovered, and in the case of maximum likelihood a single tree will have the highest
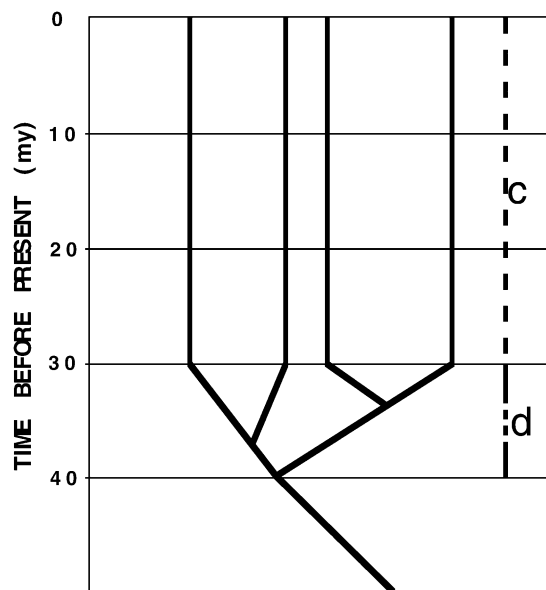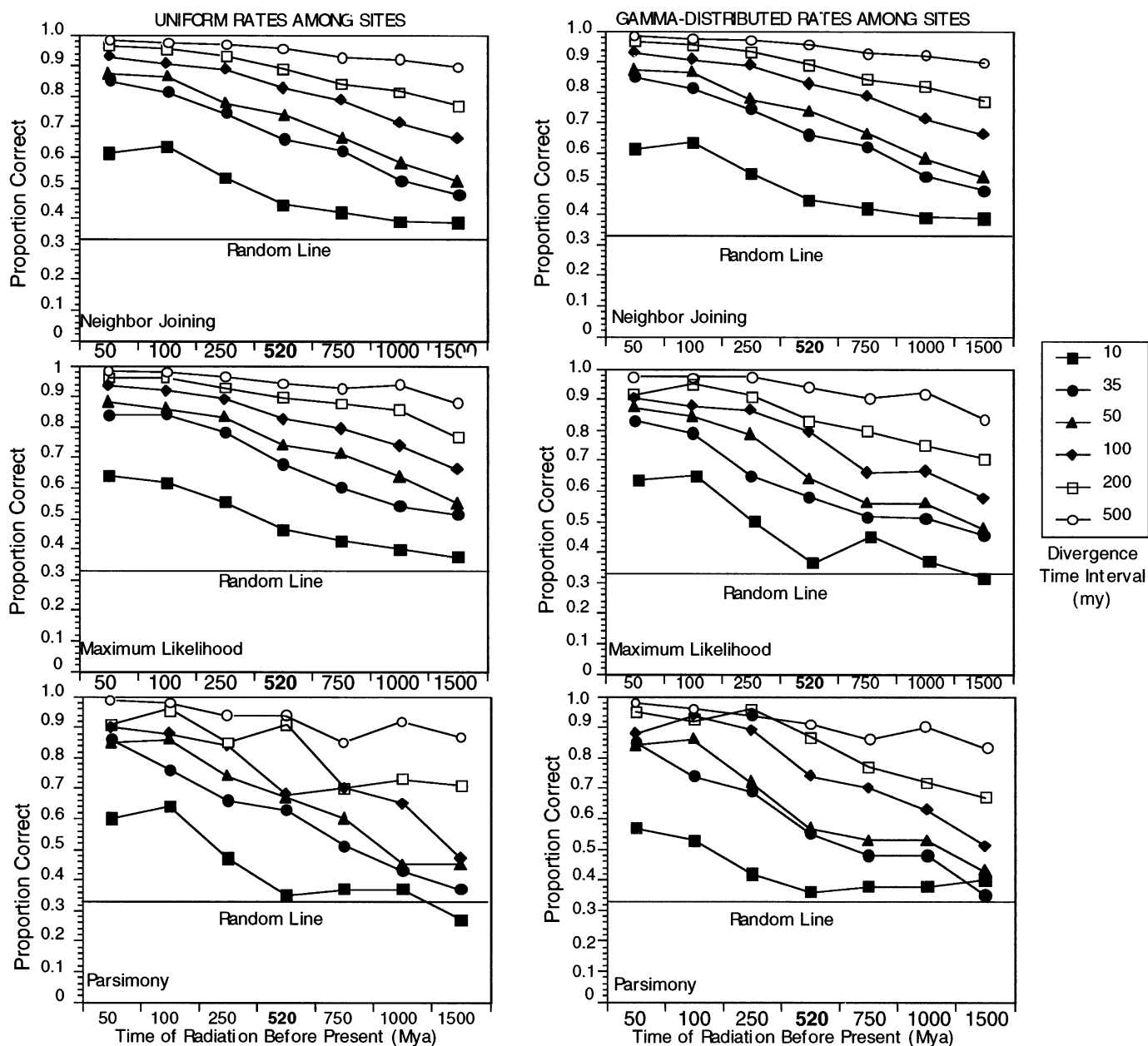
FIGURE 2—Proportion of trees that were correctly determined as a function of divergence time interval *d* and time before the present, using neighbor joining (top), maximum likelihood (middle) and parsimony (bottom). Left: Homogeneous rates of substitution. Right: Gamma-distributed rates of substitution.

likelihood. For parsimony, however, more than one tree may have the same number of steps. When this occurred, one of the equally most parsimonious trees was chosen at random. For the case of gamma distributions, Phylip was also used to resolve trees. For maximum likelihood inference of gamma distributed rate variation, rate categories were chosen as the median of the quantiles of a gamma distribution with $a = 0.5$. In all cases, we plotted the proportion of instances in which the correct tree topology was obtained.

Values of *d* included 10, 35, 50, 100, 200 and 500 m.y., ranging from the extreme Cambrian explosion hypothesis (Gould, 1989) of less than 10 m.y., to the intermediate explosion hypothesis (Valentine et al., 1996) of 35 m.y., to longer divergence times.

Values of *c* included 50, 100, 250, 520 (approximate end of Cambrian explosion), 750, 1,000 and 1,500 m.y. We report all results, but arbitrarily considered an 80 percent match between simulated trees and those inferred from simulated sequences as appropriate to be deemed "successful." For neighbor joining we simulated each condition of *d, c* with 10 replicates, each with 100 trees per replicate. We simulated each condition of *d, c* of maximum likelihood and parsimony with 100 different trees.

After these trees were constructed, their unrooted topologies were compared with the "true" tree, which we had generated. We used a program TREEDIST, which compares the proportion, *P,* of bipartite clades in common between any two trees by successively breaking up each tree at a given internode, eventually

## Performance of Techniques at 520 Mya

### Even rate distribution



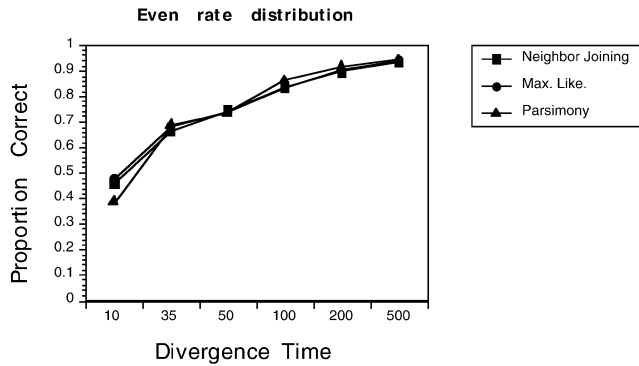FIGURE 3—Performance of the three tree-building algorithms for various values of d, at a value of c = 520 m.y.

dividing the tree into all permutations of pairs of clades. With 4-taxon trees the comparison is simple because there is only one internode in each tree. With respect to topology, the two 4-taxon trees can only differ ($P = 0$) or be topologically congruent ($P = 1$). With greater than four taxa the metric adopts a range from zero to unity.

We also investigated the ability of tree-building algorithms to resolve relationships when the substitution rate differed between the period of divergence, $d$, and the period of subsequent molecular evolution, $c$. This was done by increasing the rate above that of the previously determined optimal rate (for both periods $d$ and $c$), while keeping the rate during the subsequent divergence period at 0.0001. We also investigated the effect of increasing the number of nucleotide sites on the rate of recovery of correct trees. All other things being equal, accuracy of tree recovery should increase with number of sites, since there is more potential information stored in the longer sequences.

### RESULTS

In all simulations (Fig. 1), the radiation occurs over a time period of $d$ m.y. Molecular evolution occurs along all branches. For a subsequent time period of $c$ m.y. no more branching occurs, but molecular evolution continues according to the same scheme, which involves clocklike substitution, at a substitution rate that was optimized to recover a tree with a Cambrian divergence (see methods).

With homogeneous rates of substitution among sites (Fig. 2, left), the proportion of correct trees declines steadily with increasing values of $c$. At the Cambrian (520 m.y.a.), the success rate for the extreme Cambrian explosion hypothesis ($d = 10$ m.y.) is below 50 percent for all three techniques. With increasing time before the Cambrian, the success rate converges toward the 33 percent random expectation. Even the intermediate Cambrian explosion hypothesis ($d = 35$ m.y.) produces only modest performance in proportion of correct trees—on the order of 60–70 percent success for all three tree-building algorithms. It is only at divergence periods of $d = 100$ m.y. that proportions of correct trees consistently equal or exceed 80 percent at values of $c = 520$ m.y. or greater. There were no substantial differences in performance among the three tree-building algorithms (Fig. 3).

Simulations using a gamma distribution resulted in a poorer performance than with homogeneous rates of substitution (Fig. 2, right). As in the homogeneous rates results, a divergence time $d$ of 100 m.y. is required to obtain on the order of 80 percent success in constructing the correct tree at the time of the Cambrian. If the divergence of the animal phyla is deeper, say at 1,000 m.y.a., the success rate of divergences of 100 m.y. are generally fairly low, hovering around 60–70 percent. At deeper time periods one would probably need divergence times of 200–500 m.y. to be able to resolve 4-taxon trees with reasonable certainty of, say, 80 percent or more. In the case of gamma-distributed substitution rates, there was no substantial difference in Cambrian results among the three tree-building techniques.

The rate of nucleotide substitution and time of evolution combine to make the branch lengths of a tree. In other words, a branch length might be greater because of increased elapsed time or increased substitution rate. Therefore, it is convenient to visualize the success of tree recovery in terms of percent correct trees as a function of the ratio $c/d$ (Fig. 4). If substitution rate is constant, as is the case in our models reported thus far, then increased values of $c/d$ represent relatively briefer times of evolutionary radiation. For the branch lengths examined in Figure 2 the Cambrian explosion scenarios of 10 to 35 million years can be seen in a window of values of c/d between 52 and 15, respectively. As shown in Figure 2 the results are similar for the three tree recovery methods. The results using a gamma distribution show considerably poorer performance when c/d approaches the extreme Cambrian explosion scenario value of 52. The seemingly more erratic nature of the parsimony results may result partially from the arbitrary choice of one tree in the case of ties (trees with equal branch lengths), although ties rarely constituted more than 5–10 percent of the analyses.

Figure 5 illustrates the effect of using increased number of nucleotide sites on success of recovering the correct tree, under the two Cambrian explosion scenarios of 10 and 35 million years divergence times. We illustrate the results for neighbor joining only, because we have shown above that results do not differ substantially using the three different methods of parsimony, maximum likelihood and neighbor joining. Even with the use of 10,000 sites, the recovery success is poor. Considerably better success is obtained, however, if the substitution rate during the explosion was greater than during the subsequent time evolution (Fig. 6). For the 35 million year explosion scenario, good performance is obtained if the ratio exceeds five and good performance is obtained for the extreme explosion scenario if the ratio exceeds 8–10.

### DISCUSSION

Our results cast doubt on the likelihood of being able to resolve phylogenetic relationships if a Cambrian explosion, $d \leq 10$ million years duration, had occurred and if the molecular clock had comparable substitution rates during and since the radiation. The success level is far too low to expect any predictable and consistent degree of resolution. This is especially true for parsimony analyses using a gamma distribution of rates, which should be closer to the natural situation (Yang, 1993b). Even at the intermediate Cambrian explosion level of $d = 35$ m.y., the performance was not very good. Our results are robust with regard to method of analysis, as they do not differ using neighbor joining, maximum likelihood, or parsimony. It is only when the divergence interval is 100 m.y. or more that the success in tree building meets our criterion of 80 percent success. Given the ability of our earlier divergence time estimates (Wray et al., 1996) to resolve the order of major splits of the animal phyla, these results support the hypothesis that the divergence of the animal phyla was not explosive, but was protracted over a period of at least 100 m.y.

The only obvious way to escape these conclusions is to argue that the rate of molecular evolution was greater during the Cambrian explosion than in subsequent times. From our simulations, the rate of evolution would have to be at least five times greater to sustain the 35 m.y. divergence scenario and 8–10 times greater to support the extreme scenario of 10 m.y., if we adhere to our arbitrary criterion of 80 percent success in correct tree recovery.
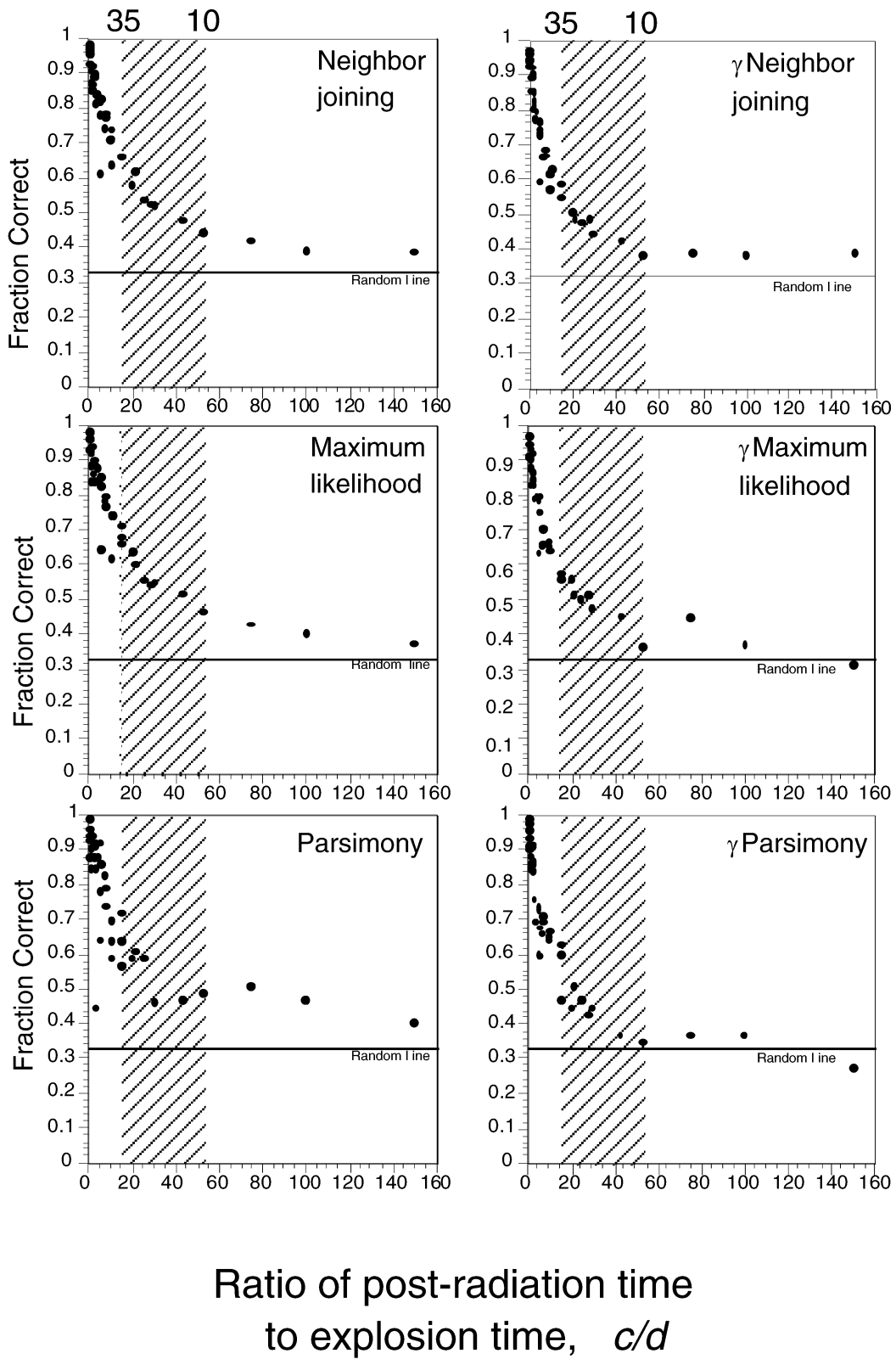
FIGURE *4*—Proportion of trees that were correctly determined as a function of the ratio *c/d,* using neighbor joining (top), maximum likelihood (middle) and parsimony (bottom). Left: Homogeneous rates of substitution. Right: Gamma-distributed rates of substitution. Shaded area includes range of *c/d* corresponding to extreme (*d* = 10 m.y.) to moderate (*d* = 35 m.y.) Cambrian explosion scenarios.
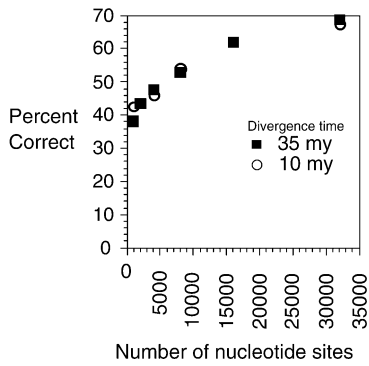
FIGURE *5*—Percent correct trees using neighbor joining as a function of the number of nucleotide sites in the sequences analyzed.



FIGURE *6*—Percent of correct trees when there is variation of the ratio of nucleotide substitution rate during period *d* to that during that of the later period *c*. Performance for divergence times of 10 and 35 million years are shown.

All simulations have simplifying assumptions that might strongly affect conclusions. We chose the branch lengths (a function of rate of nucleotide substitution and time) that would maximize the probability of recovering those correct trees whose divergences occurred at the Early Cambrian. Our birth model involves no loss of taxa. Thus our results, taking the other assumptions to be acceptable, provide a best-case scenario for recovering the correct tree. But variance in rate of evolution along branches, extinction, and departure from these optimal rates would only make the performance even poorer.

On the other hand, the use of 4-taxon trees could produce bias in the other direction. Our Cambrian explosion simulations produce long branches combined with short internodes, the latter of which correspond to the Cambrian radiation. Long branches may spuriously attract, owing to multiple hits that occur during the long time implied by these branches (Felsenstein, 1978). It has been argued that subdividing these long branches by adding taxa might improve performance, since informative sites might be preserved along one of the sub-branches (Hendy and Penny, 1989; Hillis, 1996). On the other hand, it has been pointed out that increases of taxon number can decrease performance, especially with the use of parsimony methods (Kim, 1996; Poe and Swofford, 1999). In 4-taxon cases, adding characters (nucleotide sites) should be a better strategy than adding taxa, because the probability increases of adding information that could be recovered from the relatively short internodes. The addition of slowly evolving taxa onto long branches can actually decrease recovery success, at least for parsimony analyses (Poe and Swofford, 1999). Indeed, it is not always the case that subdividing branches improves performance, because the appearance of convergence might prevent spurious attraction of long branches to other long branches (Poe and Swofford, 1999). Nevertheless, we are now in the process of extending our model to understand the addition of branches.

It is also important to understand the effect of increase of number of taxa on performance, especially because we probably have over 30 phyla that originated in the Cambrian, that can only be subdivided into three major clades, the Ecdysozoa, Lophotrochozoa, and Deuterostomia (Aguinaldo et al., 1997; Balavoine and Adoutte, 1998). Unfortunately, measures of tree comparison become more complex when trees with more than four taxa are compared. With four taxa, two trees can either be the same or different in topology, but larger trees require a measure of similarity that is inherently arbitrary. Our comparison program, TREEDIST, calculates the number of bipartite clades in common between the simulated tree and the tree recovered from the sequences at the tree tips. Such a measure ignores whether the differences are concentrated at the tips or towards the base. Another
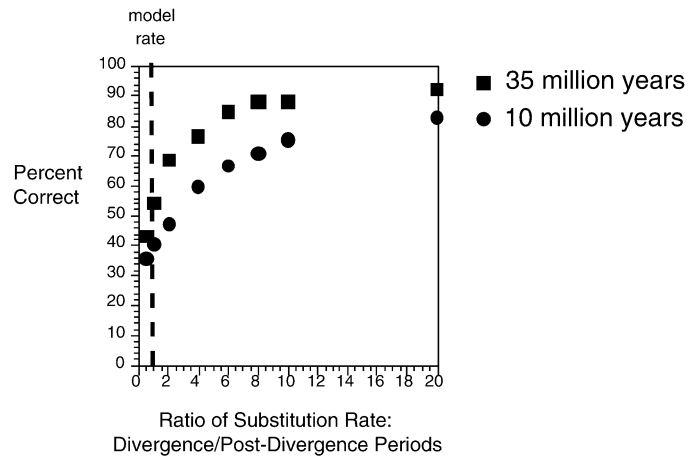
approach might be to compare the branch lengths between all taxa in the simulated trees and trees constructed from the sequences. We are also now investigating these measures. A preliminary attempt, using 25 taxa, shows that the TREEDIST measure produces discouraging results for a Cambrian explosion scenario (Fig. 7). The proportion of correct groups for the two Cambrian explosion scenarios recovered by maximum likelihood is less than 25 percent and is less than 15 percent for neighbor joining. This result may be mostly a function of the rigorous requirement of exact match of clade membership. But it also may indicate why studies on the large scale level of multiphylum relationships can be a tricky enterprise.

The difficulty of inferring evolutionary trees during the divergence of the animal phyla has been a subject of extensive investigation, and it has been noted previously that an explosion might preclude our ability to resolve the evolutionary relationships of the animal phyla (Philippe et al., 1994; Abouheif et al., 1998). Despite the difficulties, some measure of success and consistency has been obtained in grouping the phyla into subgroups with molecular data (Halanych, 1995; Aguinaldo et al., 1997; McHugh, 1997; Winnepenninckx et al., 1995). Our study provides insight
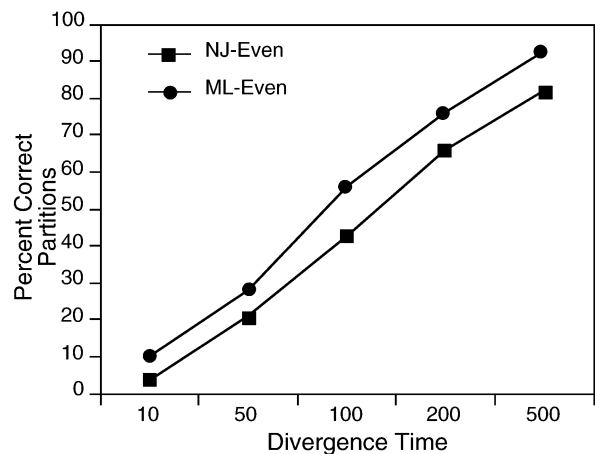


FIGURE *7*—Percent of correct bipartite clade partitions between simulated trees and trees inferred from simulated sequences using neighbor joining, for trees of 25 taxa, over a range of values of *d*.

into why it may be so difficult to obtain such resolution. More research is required to investigate the role of differential rates of molecular evolution along different branches, the effects of different number of taxa and nucleotide sites in resolving trees, and the use of different criteria of judging the success of phylogenetic reconstruction.

The timing of divergences may have great significance, as they also define the rise of morphological, physiological, developmental, and genetic characters associated with the synapomorphies of particular groups. It was claimed (Gould, 1998) that it does not ''matter one whit'' whether one line of wormlike organisms carried the line leading to the animals across the Cambrian boundary or if ''10 similar wormlike species already representing the lineages of 10 subsequent phyla'' crossed the Cambrian chasm. This statement ignores the important point that the ancestor bore the characters known to be homologous in the sister taxa that comprise the descendants. In the case of the protostome-deuterostome split, this entails: The presence of a set of *Hox* genes, nervous system, muscular system, presence of gene for lysyloxidase (that in the presence of molecular oxygen, crosslinks collagen triple helices to produce ligaments and tendons), antero-posterior determination including anterior concentration of nerves, the *Pax-6* gene (associated with vision), rhodopsin, and the muscle-specific *myo-D* gene (turns on muscle fate) (DeRobertis and Sasai, 1996; Levinton, 2001; Ohno, 1996). These characters give us the following picture of the protostome-deuterostome ancestor: a bilaterian creature with nervous organization, cephalization, the capability of large body size, owing to a circulatory system, and the capability of producing a skeleton, owing to the presence of lysyloxidase. In other words, even this crude an analysis gives us a bilaterian that can take the form of many of the body plans of the modern animal phyla. If divergence times *within* the protostome or deuterostome phyla are similarly found to be in the middle Proterozoic, then even more specific characters will define ancestral states deep in Precambrian time. While it is possible that these states still may define organisms that are not clearly morphologically differentiated as may happen in early stages of radiations (Radinsky, 1982) the data are also compatible with the alternative possibility that the characters that fully distinguish the phyla were accompanied by a hidden Precambrian array of differentiated morphologies, perhaps associated with small body size. The recent discovery of wormlike trails of macrobenthic size, approximately one billion years old or greater (Seilacher et al., 1998; Rasmussen et al., 2002), should at least give us pause about closing the books on the possibility of the evolution of the bilateria at a time much before the Cambrian.

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