Testing Phylogenetic Approaches with Empirical Data, as Illustrated with the Parsimony Method

Marc W. Allard and Michael M. Miyamoto
Department of Zoology, University of Florida

In the absence of certainty, well-supported phylogenies stand as our best estimates of the correct evolutionary relationships for a group (Miyamoto and Cracraft 1991). Such phylogenies provide a solid foundation for understanding the origins and history of biological diversity and the forces responsible for them. However, what remains less obvious is that they can also serve as standards against which the results of different tree-building procedures can be compared to determine their accuracy. Approaches that lead to solutions congruent with well-supported topologies should be preferred over those that rarely do so. Thus, well-supported phylogenies fill the role of model trees in simulation studies, as the basis for comparison of phylogenetic reliability (Nei 1991; also see Mickevich 1978).

In the present study, the evolutionary relationships of lipotyphlan insectivores (class Mammalia, infraclass Eutheria) are investigated with new mitochondrial DNA (mtDNA) sequences of the 12S ribosomal RNA (rRNA) gene. A single phylogeny based on parsimony analyses of these sequences is accepted as well supported according to different criteria, although an exception to this conclusion is noted. This exception forms the basis for an investigation of why an incorrect solution is obtained by the parsimony method in this particular case.

The 12S rRNA gene sequences for the lipotyphlans *Amblysomus hottentotus* (AHO; African golden mole, family Chrysochloridae), *Atelerix albiventris* (AAL; hedgehog, Erinaceidae), and *Blarina brevicauda* (BBR; short-tailed shrew, Soricidae) were determined by a combination of the polymerase chain reaction (PCR) amplification and direct dideoxy sequencing of the asymmetrical products (Allard et al. 1991). These new sequences were aligned to each other and to outgroup representatives from three other eutherian orders: *Bos taurus* [BTA; domestic cow, Artiodactyla (Anderson et al. 1982)], *Homo sapiens* [HSA; human, Primates (Anderson et al. 1981)], and *Rattus norvegicus* [RNO; rat, Rodentia (Gadaleta et al. 1989)]. These orthologues were chosen to provide alternative mammalian outgroups which have had a widespread use in comparative molecular studies. In the final alignment of the study group and outgroups (fig. 1), gaps were included only when they saved two or more substitutions (Kraus and Miyamoto 1991). Throughout the present study, gaps were treated as single differences regardless of their lengths.

Parsimony analyses of the 12S rRNA sequences were conducted with the computer
FIG. 1.—Final alignment of the 12S rRNA gene sequences for AHO, AAL, and BBR and for HSA, BTA, and RN0 (the study group and outgroups, respectively). Dashes refer to gaps which were introduced to maintain the overall alignment. The sequence for AHO is shown in its entirety. For the other species, only their gap positions and those nucleotides differing from AHO are shown. The two overscored regions (positions 311-376 and 883-933) correspond to blocks of the 12S rRNA gene that are highly variable and therefore difficult to align (Allard 1990). The new sequences for lipotyphlans have been deposited in GenBank under accession numbers M95108-M95110.
program PAUP (Swofford 1990). For phylogenetic reconstructions, the 12S rRNA orthologues were treated six different ways according to outgroup (BTA, HSA, or RNO) and mutation type [transitions, transversions, and gap events (ALL) versus the more slowly evolving transversions only (TV)]. Outgroups were included separately to permit more detailed examinations of the most-parsimonious trees and their alternatives.

The one exception to the phylogeny, where the topology accepted as well supported was not found, occurred with BTA as outgroup and TV alone. To investigate why a different solution was supported by parsimony in this case, we optimized the TV data (with BTA as outgroup) onto the accepted (AHO,BBR) arrangement. In these optimizations, ambiguous TV (i.e., those with more than one most-parsimonious placement) were assigned to the (AHO,BBR) phylogeny such that each allowable reconstruction was given the same weight (Swofford and Maddison 1987; Maddison and Maddison, accepted; also see Fitch 1971). For the three lipotyphlans and BTA, this approach resulted in a more equal distribution of ambiguous change among the terminal branches of the accepted arrangement.

The optimized branch lengths of this topology were then converted into probabilities of transversional change (TV/positions observed to have changed by TV). The denominator for these probabilities was estimated as the number of positions with a transversion, as determined from an expanded sequence file with additional representatives of Artiodactyla [Capra hircus (Kraus and Miyamoto 1991)], Primates [Pongo pygmaeus (Hixon and Brown 1986)], and Rodentia [Mus domesticus (Bibb et al. 1981)]. Sites not changed by TV were excluded from the denominator to provide more accurate estimates of the frequencies of change and the influences of mutational saturation (Shoemaker and Fitch 1989). Average probabilities for the two longest branches and for the three shortest (including the internal branch) were calculated (as P and Q, respectively) and were compared with the analytical results of Felsenstein (1978, 1983), summarizing when parsimony becomes positively misleading (i.e., inconsistent).

In five of six cases, the most-parsimonious tree favored an (AHO,BBR) sister-group relation (table 1). In two of these instances (ALL and TV, with RNO as outgroup), the support for (AHO,BBR) was significant (P < 0.05) according to the binomial test of Williams and Goodman (1989). These results are congruent with the most recent and thorough analyses of morphological data for insectivores (Novacek 1986; Butler 1988). Thus, an (AHO,BBR) relationship can be accepted as well supported on the grounds of stability, statistical significance, and congruence.

Acceptance of the (AHO,BBR) arrangement as well supported requires that the single exception in favor of (AAL,AHO) (with BTA as outgroup and with TV only) is incorrect (table 1). Why, then, is an incorrect solution obtained by parsimony in this particular case? Insights into this question come from two lines of evidence. The first line is that the incorrect (AAL,AHO) solution is defined by a significantly greater number of informative TV than is the least-parsimonious, other incorrect arrangement for these insectivores [(AAL,BBR); 24 vs. 8, respectively; binomial test for equal support; P < 0.01] (Penny et al. 1991). The transversional support for (AAL,AHO) is therefore unlikely to be the result only of sampling error.

The second line of evidence is that the average probability of change for AAL and AHO (P = 0.23) is twice as great as that for BBR, BTA, and the internal branch (Q = 0.11) when calculated according to the accepted phylogeny (fig. 2A). The single exception in favor of (AAL,AHO) therefore represents a parsimony solution in which
Table 1
Numbers of Unique Informative Positions Supporting the Three Possible Dichotomous Arrangements for the Three Lipotyphlans and an Outgroup

<table>
<thead>
<tr>
<th>Dichotomous Arrangement</th>
<th>No. of Unique Informative Positions(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSA</td>
</tr>
<tr>
<td></td>
<td>ALL</td>
</tr>
<tr>
<td>AHO (\underset{BBR}{\longrightarrow}) (\underset{OUT}{\longrightarrow})</td>
<td>22</td>
</tr>
<tr>
<td>AAL (\underset{BBR}{\longrightarrow}) (\underset{OUT}{\longrightarrow})</td>
<td>31</td>
</tr>
<tr>
<td>AHO (\underset{AAL}{\longrightarrow}) (\underset{OUT}{\longrightarrow})</td>
<td>18</td>
</tr>
</tbody>
</table>

\(a\) OUT = BTA, HSA, or RNO.
\(b\) TV were scored by recoding the nucleotides as purines or pyrimidines. Thus the numbers for TV sometimes exceed those for ALL, since some uninformative positions with three or four bases become informative after being recoded as two-state characters [e.g., site 141 with ATAC for AHO, AAL, BBR, and BTA is uninformative but becomes informative when rescored as RYRY, respectively (fig. 1)].

The two longest branches are joined. In the calculations of these probabilities, the number of positions changeable by TV is taken as 261, as estimated from the expanded sequence file (see above). In its support, this estimate (261 sites of 985 positions, or 27% of the total gene region for nine species) is similar to that for the six sequences in figure 1 (236) and to the level of divergence (=23%) at which 12S rRNA gene sequences for mammals begin to saturate (Mindell and Honeycutt 1990).

The accepted topology with BTA as outgroup and with TV only is similar to the Wagner parsimony model used by Felsenstein (1978, 1983), in terms of its number of taxa (four) and characters (two-state, reversible attributes) (fig. 2A). The assumption of an equal probability of change for all sites is approximated by counting only positions changeable by TV. When plotted against Felsenstein's results, the \(P\) and \(Q\) estimates for the accepted topology fall within the region of consistency but close to the boundary of inconsistency (fig. 2B). When considered with the other lines of evidence, this plot suggests that the (AAL,AHO) solution, for this set of sequences, is related to both stochastic error and the greater opportunities for chance similarity between the longest branches. The greater probability of change for the two longest branches introduces some attraction, which alone is insufficient to explain the support for (AAL,AHO).

However, this attraction makes it easier to obtain the incorrect (AAL,AHO) phylogeny, because of stochastic error which is inherent in any sample of characters. These results are expected to converge onto the accepted (AHO,BBR) arrangement as new data are added, a prediction which follows from the consistency of \(P\) and \(Q\) (fig. 2B). The same conclusions are also suggested when the transformational probabilities of figure
Fig. 2.—A, Accepted arrangement for lipotyphlans, with probabilities of change shown for each branch. This reconstruction is based on transversions only, with BTA as outgroup. Branch lengths are proportional to the probabilities of change. B, Transformational probabilities for the two long branches (P) and three short internodes (Q) of the model phylogeny used by Felsenstein (1978, 1983) to test when parsimony becomes inconsistent. Values of P and Q to the left of the curve represent instances where parsimony is positively misleading. X = P and Q values for the accepted phylogeny (A), as averaged for its two longest (AAL and AHO) and three shortest (BBR, BTA, and common) branches, respectively.
2A are corrected for multiple TV, according to the method used by Kraus and Mi-
yamoto [1991, table 2; Tajima and Nei (1984)].

The explanation that the attraction of the longest branches is at least partly re-
sponsible for the incorrect (AAL,AHO) arrangement is strengthened by the results of
analyzing the three outgroups together and of removing from consideration the highly
variable regions of the 12S rRNA gene (Swofford and Olsen 1990). When the three
outgroups are combined and TV alone are counted, HSA and RNO both join to the
AAL branch, thereby breaking it up and reducing its tendency to unite with AIIO,
the other long branch. As a result, the accepted (AHO,BBR) arrangement becomes
further supported by BTA, which now joins to AAL rather than to BBR (see table
1). This solution is also obtained when the highly variable regions of the 12S rRNA
gene are removed [positions 311–376 and 883–933 (fig. 1); also see table 2 and below].
Removing these regions reduces the overall probabilities of change (i.e., P and Q),
thereby making it less likely for homoplasy to occur even between the longest branches
(fig. 2B).

Despite being more prone to saturation (Mindell and Honeycutt 1990), the im-
portance of TV (and to a lesser extent, gap events) at these hierarchical levels is
supported by the results for all mutations and BTA as outgroup (table 1). In contrast
to the situation with TV only, the accepted (AHO,BBR) arrangement is obtained
when all mutations are counted, with the strongest evidence coming from transitions
and the one informative gap event (table 2). Only 8% of these mutations originate
from the variable regions of the 12S rRNA gene, in contrast to 41% for TV (the
difference is significant; χ² test of independence with Yates's correction: χ² = 7.25;
df = 1; P < 0.01). For conserved regions, the transition/transversion ratio for the
three lipotyphlans and BTA averages 1.24 (standard error = 0.16), but the ratio is
only 0.65 for the variable regions (standard error = 0.10). The latter ratio is similar
to that (0.47) expected for fully saturated sequences with equilibrium base frequencies
of 0.36 A, 0.20 C, 0.18 G, and 0.26 T (i.e., frequencies in the study group and BTA)
(Holmquist 1983). In saturated regions, TV erase transitions as the former accumulate.

Table 2

<table>
<thead>
<tr>
<th>Dichotomous Arrangement</th>
<th>No. of Transitions + Gap Events in Conserved Regions</th>
<th>No. of TV in Conserved Regions</th>
<th>No. of Transitions + Gap Events in Variable Regions</th>
<th>No. of TV in Variable Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHO [BBR ]</td>
<td>5</td>
<td>13</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>AAL [BTA ]</td>
<td>11</td>
<td>14</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>AHO [AAL ]</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>BBR [AHO ]</td>
<td>7</td>
<td></td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

NOTE.—Only a single gap event from a conserved region and favoring (AHO, BBR) is represented.
in these unreliable areas (Brown et al. 1982). In the process, transitions become limited to conserved regions, thereby enhancing their phylogenetic reliability. According to this explanation, one would therefore be better off weighting the conserved versus variable regions of the 12S rRNA gene instead of its transitions and TV.

In this study, the success of the parsimony method to identify a well-supported phylogeny reflects favorably on its phylogenetic efficiency. This efficiency was next examined for other phylogenetic approaches by using the same data. The orthologues for the study group and BTA (with both the conserved and variable regions) were analyzed by the evolutionary-parsimony (Lake 1987), neighbor-joining (Saitou and Nei 1987), and maximum-likelihood (Felsenstein 1981) methods, using substitutions and/or TV alone. For the neighbor-joining method, pairwise distances were corrected either by the Kimura (1980) procedure (for substitutions) or as described above (for TV only). With substitutions, the accepted (AHO, BBR) arrangement was obtained by both the neighbor-joining and maximum-likelihood methods. In contrast, both of these procedures supported the incorrect (AAL, AHO) topology when only TV were counted. The same is true for the evolutionary-parsimony procedure, which gains its phylogenetic information from TV even though substitutions are counted. Thus, by emphasizing TV, all three of these approaches converged onto the same incorrect arrangement as did the parsimony method (table 1). As before, for transversion parsimony, these results can now serve as the basis to investigate the behavior of these other methods.

Tests of phylogenetic reliability, relying on well-supported phylogenies and empirical data, complement the work of simulation analyses in that the former exemplifies the real world, whereas the latter offers greater control over the parameters and conditions that determine the success of each method. Thus, the preferred strategy to elucidate the strengths and limitations of different procedures remains one that integrates both approaches.

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