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Advantages and Disadvantages of Molecular Phylogenetics: A Case Study of Ascaridoid Nematodes[1]

S. A. NADLER[2]

Abstract: The advantages of nucleotide sequence data for studying phylogeny have been shown to include number of potential characters available for comparison, rate independence between molecular and morphological evolution, and utility of molecular data for modeling patterns of nucleotide substitution. Potential pitfalls have also been revealed and include difficulties of inferring positional homology, incongruence between organismal and gene genealogies, and low likelihood of recovering the correct phylogeny given certain patterns in the timing of speciation events. Statistical methods for comparing phylogenetic hypotheses have been used to assess the reliability of alternative trees for ascaridoid nematodes. Based on partial ribosomal RNA sequences, tree topologies inconsistent with monophyly of the Ascaridinae were significantly worse by maximum likelihood inference. The topology of the maximum parsimony tree based on full-length sequences of 18S rRNA and 300 nucleotides of Cytochrome oxidase II for 13 ascaridoid species was generally consistent with traditional taxonomic expectations at lower ranks, but inconsistent with most proposed arrangements at higher taxonomic levels.

Key words: ascaridoid, cytochrome oxidase, molecular systematics, phylogenetics, ribosomal-RNA.

During much of this century, procedures for estimating phylogenetic relationships have been thought to lack the level of objectivity characteristic of other analytical scientific methods, and, until recently, phylogenies were regarded as having little utility beyond the construction of genealogically correct classifications. During the last decade, advances in both molecular and phylogenetic methods have dramatically changed this common viewpoint. Inferred evolutionary trees (phylogenetic hypotheses) are now recognized as forming the necessary framework for comparative study of a wide spectrum of subjects both within and outside the traditional boundaries of evolutionary biology (1,2,8). For example, phylogenetic studies have been used to infer the genealogies of genes comprising larger multi-gene families (13,28), investigate rates of molecular evolution (6), and trace the molecular epidemiology of infectious diseases (15). Substantial theoretical advancements in methods of inferring phylogenetic trees have followed the growth of molecular systematic studies (10, 16,21). This growing body of information has revealed some of the implicit assumptions underlying different tree inference methods, and, for simple cases, the conditions under which particular tree-building procedures are likely to yield erroneous results. Additional theoretical modeling is required to understand potential pitfalls of tree estimation procedures for larger data sets with more complicated components (10,21). Clearly, much remains to be studied concerning methods of phylogenetic inference based on nucleotide sequence data. As summarized by Felsenstein (4), inferring evolutionary history should be viewed as "making an estimate of an unknown quantity, in the presence of uncertainty, and using a probabilistic model of the evolutionary process." Given that the level of confidence in any individual esti-

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[2] Associate Professor, Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115-2861.

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mate may be difficult to quantify, phylogenetic trees should be viewed as provisional hypotheses that may change pending acquisition of additional data or application of improved analytical procedures.

Advantages and Disadvantages of Sequence Data

Advantages: One major advantage of sequence data is the large number of potential characters available for inferring relationships. The number of nucleotide characters that can be obtained efficiently for comparative evolutionary studies has grown tremendously since the introduction of the Polymerase Chain Reaction (PCR) in 1985 (22). In theory, the amount of available data for nucleotide-based studies of evolutionary relationships is limited only by the number of homologous and independent characters for the taxa investigated. In practice, many coding and non-coding sequence regions may be relatively uninformative about evolutionary relationships due to inappropriate rates of nucleotide substitution for the time scale of the speciation events studied.

A second advantage of nucleotide-based studies is that substitutions within structural genes are decoupled from changes in morphology (29). It is undisputed that morphological evolution has a genetic basis. However, substitutions in the structural loci typically sequenced in phylogenetic investigations are not expected to influence the external phenotype. Therefore, orthologous molecular comparisons can be useful for phylogenetic investigation when morphological features are not (for example, when rapid morphological evolution makes determination of structural homologies difficult or, conversely, when morphological change is so conservative that few characters are variable among the ingroup species).

A third advantage of nucleotide data is that information from the sequences themselves can be useful for specifying parameters of the model of sequence evolution, which, in turn, influences the topology of the inferred tree. Thus, information on the likelihood of different classes of substitution events for a particular nucleotide sequence can be valuable for choosing the most appropriate model of sequence evolution and estimating the tree topology. For example, recent empirical studies on the accuracy of phylogenetic estimates (10,21) have demonstrated that methods that incorporate information on expected differences in the frequencies of transition versus transversion substitutions yield more accurate estimates of evolutionary history.

Disadvantages: Prior to any evolutionary analysis, orthologous sequences from different species must be aligned to establish homology by nucleotide position. Inferences of positional homology are critical for all subsequent analyses of tree structure and reliability, and for more commonly reported features of sequence comparison such as percent identity. Inferences of positional homology are frequently more problematic for non-coding nucleotide sequences because penalties for insertion-deletion events determine the extent of sequence similarity during pairwise and multiple alignment. The introduction of unlimited gaps would allow any two sequences to match perfectly; therefore, the outcome of a multiple alignment depends on the gap penalty values applied. Unfortunately, the utility of a particular gap penalty value must be assessed a posteriori, and not from factors intrinsic to the sequences. Thus, computer-assisted multiple alignments are often based on arbitrarily assigned gap penalties that are then uniformly applied to the sequences. With high rates of nucleotide substitution or insertion-deletion events, confidence in positional homology for certain regions of non-coding sequence may be low, and these regions may need to be excluded from the phylogenetic analysis. In contrast, a significant advantage of protein-encoding gene sequences is that the amino acid sequence of the polypeptide is usually more conserved than the underlying nucleotide sequence due to degeneracy of the genetic code and functional constraints on amino acid substitutions. As a result, multiple alignment of protein coding nucle-

otide sequences is best performed on the deduced amino acid sequence, and inferred loss or gain of individual amino acids can then be used to place gaps corresponding to codon loss or gain.

Molecular phylogenies are usually based on comparison of sequences representing a single orthologous gene from many species. A potential shortcoming of this approach is that the evolutionary history of species can be incongruent with the genealogy of a single gene (19). Because intraspecific polymorphism is a common feature of populations, a large proportion of genetic loci will include multiple alleles that have arisen in an ancestor-descendant manner from pre-existing alleles. Thus, the origin of extant alleles in ancestral taxa may predate the speciation events that yielded the organisms in which the alleles are currently found. Whether a gene genealogy is congruent with the pattern of organismal speciation depends on the pattern of allele sorting and loss over time. In the absence of independent evidence, it is not possible to assess if a tree topology based on a single gene sequence is likely to represent the genealogy of the species. By contrast, when independently derived trees representing two or more unlinked gene loci show congruent topologies, the organismal phylogeny can be accepted with greater confidence. Similarly, congruence between genealogies inferred from independent analyses of mitochondrial and nuclear genes most likely results from the common cause of speciation over time.

Phylogeny estimation benefits from studying molecules with rates of change that are appropriate given the timing of the speciation events. However, it is not always possible to produce reliable estimates of evolutionary history using molecular data. Both Nei (19) and Lanyon (14) modeled the effects of time of shared versus independent ancestry (Fig. 1) within a phylogeny on the probability of recovering the correct tree with different rates of nucleotide substitution. In summary, rapidly evolving gene sequences may retain information on the correct tree topology if the time of independent ancestry for sister

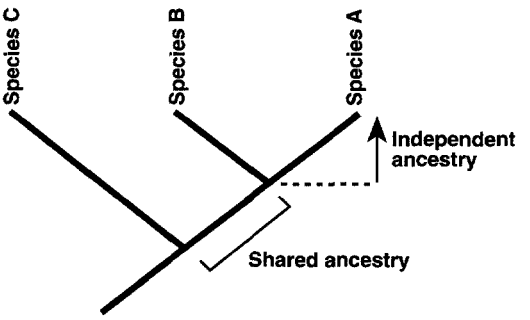

FIG. 1. Hypothetical tree depicting periods of shared and independent ancestry for species A and B. Differences in the relative lengths of time corresponding to the internal branch (shared ancestry) and the external nodes or terminal branches (independent ancestry) leading to species A and species B affects the probability of recovering the correct tree topology with molecular data.

taxa is short. Conversely, if this time is long, shared-derived character states inherited from the common ancestor of the sister taxa are likely to be lost due to multiple substitutions at sites that were once phylogenetically informative. If the period of shared ancestry is short, slowly evolving sequences are likely to be uninformative because the probability of a substitution leading to evolutionary information (a shared-derived character) is low, regardless of the time period of independent ancestry. In the scenario where the time of shared ancestry is short and independent ancestry is long, most sequences are likely to be depauperate in sites that are informative about the correct topology. These examples show that there are real evolutionary scenarios where nucleotide sequence data are unlikely to be useful in recovering organismal genealogy.

PHYLOGENETIC INFERENCE

Considerable debate has been focused on the relative merits of different methods of estimating phylogenetic relationships from nucleotide sequence data (21,27). Recent theoretical and empirical studies of tree inference methods have demonstrated the advantages and disadvantages of different approaches, and have shown that there is no method of inference that can be universally applied to produce error-free trees (21). For this and other reasons, phylogenies are best regarded as

"working hypotheses" because the depicted relationships may be subject to revision as more appropriate methods of analysis (models) become available. Although all current molecular approaches have potential drawbacks, nucleotide sequence data remain advantageous in that theoretical and empirical advances in molecular evolution contribute to the ability to specify an appropriate model of substitution. From a practical standpoint, molecular systematists need inference methods that recover the correct tree under the ideal model of substitution (consistency) and also with significant deviations from the model (robustness). This is a particularly challenging area for future research (21).

Methods of phylogenetic inference that employ some criterion of optimality in evaluating alternative trees, such as maximum parsimony or maximum likelihood, are characterized by a fundamental difficulty in that large numbers of different trees must be compared. For example, for five taxa there are 15 different rooted bifurcating trees, whereas for 10 taxa there are more than 2×10^6. Although evaluating an optimality criterion for a single tree is simple, searching for the optimal tree(s) among the set of all trees can be extremely difficult; in fact, there is no computationally efficient algorithm for solving this problem (27). Given this difficulty, nonexact (heuristic) search methods are often used for studies of moderate numbers of taxa, and although such algorithms usually perform well, they may fail to recover the tree(s) that best fit the optimality criterion. Another difficulty is that different methods of inference utilize different amounts of potential information in the sequences themselves. For example, methods based on pairwise genetic distances use fewer of the available data as the number of taxa (n) is increased because the number of pairwise distance values in the matrix increases as a function of n^2, whereas the number of different trees to be evaluated increases exponentially.

When a molecular investigation yields a single best tree (point estimate), investigators are faced with assessing the reliability of this result. Although tests of reliability such as bootstrapping cannot be equated with accuracy, assessment of the relative level of support for various clades within the tree is warranted prior to systematic interpretation. In addition, analytical tests are available to determine if alternative tree topologies are significantly worse than the point estimate. One caveat for resampling methods and analytical tests is that these approaches are dependent on the assumptions of the particular inference method used and the limitations of the available data. For example, if the gene tree under investigation is incongruent with the history of the species, then a reliably supported topology will be positively misleading with respect to the species phylogeny. Likewise, even when alternative trees are significantly worse than the point estimate, it is possible that the point estimate is incorrect and the alternatives are simply worse given the available evidence.

Molecular Systematics of the Ascaridoidea

Secernentean nematodes of the superfamily Ascaridoidea are common gastrointestinal parasites hosted mainly by carnivorous vertebrates occupying terrestrial and aquatic habitats. In the most commonly used classifications, four (3,5) or five (7) families are recognized. Most of the approximately 60 accepted genera are distributed between the families Ascarididae and Anisakidae. The vast majority of ascaridoid diversity is found among marine fish, crocodilian, snake, and terrestrial mammalian hosts (25). Relatively few species parasitize hosts that are normally herbivorous, and in such cases development from juvenile to adult can usually be completed within the definitive host following ingestion of embryonated eggs. By contrast, the majority of ascaridoids that have been characterized require intermediate or transfer hosts to complete their life cycles. Species in the subfamilies Ascaridinae and Toxocarinae, which are principally parasites of terrestrial mammals, are responsible for diseases in domesticated ani-

mals and wildlife. *Ascaris lumbricoides* is the causative agent of human ascariasis, and, as juveniles, other ascaridoid species are responsible for human diseases such as visceral larva migrans and anisakiasis.

It has proved difficult to define a large number of homologous morphological characters for phylogenetic analysis of ascaridoid species. Fagerholm (3) has compiled the available information, which includes data on 22 morphological characters from species representing 47 of the described genera. Unfortunately, the utility of these data for assessing phylogenetic relationships has not yet been evaluated. Published evolutionary hypotheses for ascaridoid species usually have been based on an investigator's emphasis of one or few "key" morphological features or life cycle characteristics (5,11,20,24,25). These proposals conflict with respect to relationships among higher taxa, and hypotheses for the relationships of genera within subfamilies are virtually nonexistent. Protein-electrophoretic and immunological data have also been of limited usefulness for phylogenetic inference due to the high level of genetic differentiation between congeners (17). By contrast, partial sequences of 18S and 28S ribosomal RNA have been shown to have utility for estimating phylogenetic relationships for representative ascaridoid taxa (18). Herein, the results of previously published results are reviewed, and the usefulness of both full-length 18S rRNA and partial cytochrome oxidase II (mitochondrial) sequences are explored for a greater diversity of ascaridoid taxa.

In a previous study (18), a data set consisting of 395 sites of ribosomal RNA sequence, including 18S and 28S regions, was compared for eight ascaridoid species and rooted using 18S sequence for the outgroup *Caenorhabditis elegans*. Seven ingroup taxa used in this analysis were from the Ascarididae, four species from the Ascaridinae (*Ascaris suum, Parascaris equorum, Baylisascaris procyonis,* and *B. transfuga*), two from the Toxocarinae (*Toxocara cati* and *T. canis*), and one from the Heterocheilinae (*Heterocheilus tunicatus*). The other ingroup species (*Terranova caballeroi*) was the only specimen considered to represent a different family (Anisakidae), sensu Gibson (5).

These sequence data were analyzed using two distinct character-state approaches—maximum parsimony (MP) and maximum likelihood (ML). Seventy-nine of the 395 sites in the aligned sequences were phylogenetically informative by the minimum substitution method of MP. Analysis of ascaridoid taxa by MP resulted in a single minimum-length tree of 226 steps (Fig. 2). Bootstrap resampling coupled with MP analysis recovered the identical topology and suggested that many of

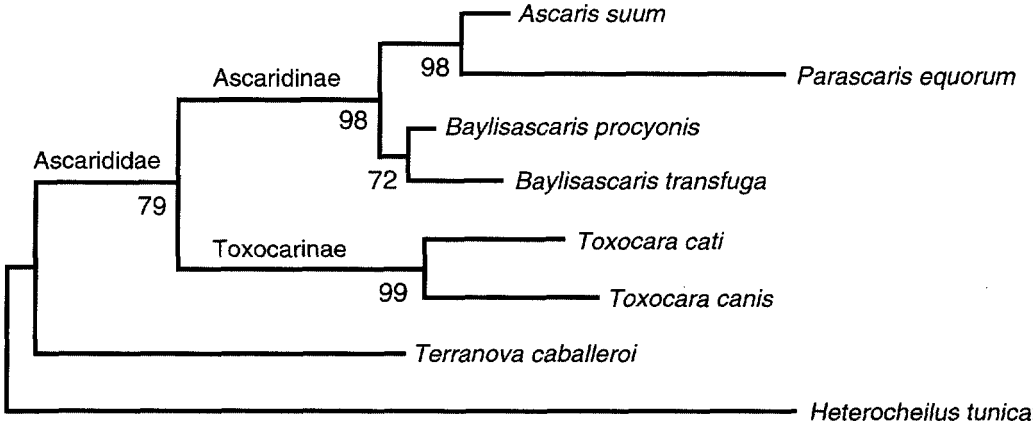

FIG. 2. Maximum parsimony (MP) tree based on analysis of 395 sites of 18S and 28S sequence (18). Bootstrap percentages of clades are shown below internal nodes. Branch lengths are scaled to the number of substitutions inferred by parsimony analysis. This tree was rooted based on MP analysis of partial 18S data, with *C. elegans* as the outgroup (18). These sequences are deposited as GenBank accession numbers M90441-M90456.

the clades within the tree received a reasonable level of support (Fig. 2). For example, species representing the Ascaridinae and Toxocarinae were monophyletic in more than 95% of the bootstrap MP replicates. The topology recovered by the MP analyses was very similar to that obtained by ML analysis, in which all aligned sites were evaluated according to a model of substitution in which the expected rate of transitions was twice that of transversions. The ML tree (Fig. 3A) differed from the shortest MP tree (Fig. 3B) only with respect to monophyly of the *Baylisascaris* species. Based on overall morphological similarity, species in the genera *Ascaris* and *Baylisascaris* are traditionally considered to be closely related. Therefore, the high level of bootstrap support for the clade consisting of *P. equorum* and *A. suum* was unexpected. To determine if traditional arrangements of taxa and other alternative evolutionary hypotheses were significantly worse, competing trees were tested statistically by the ML method of Kishino and Hasegawa (12). This analytical approach is useful for determining which groupings in the tree are reliably supported, given the assumptions of the inference method and the limitations of the available data. For example, for these data, the MP tree (Fig. 3B) was not significantly worse than the optimal ML tree (Fig. 3A) as assessed by the mean and variance of log-likelihood differences between trees (18). Likewise, the traditional expectation of an *Ascaris* plus *Baylisascaris* clade (Fig. 3C) was not significantly worse than the topology depicting a *P. equorum* plus *A. suum* clade (Fig. 3A). This shows that although these data yield a single point estimate by ML inference, the depicted relationships for the Ascaridinae were not strongly supported in a statistical sense. In contrast, alternative trees that did not preserve the monophyly of the Ascaridinae or Toxocarinae were significantly worse (e.g., Fig. 3D), as were arrangements that separated congeners (18). These statistical tests suggest that these rRNA data are most informative for deeper branches in the tree, and this interpretation was also supported by the number of inferred substitutions for internal branches of the MP and ML trees (18).

Recently, the utility of full-length 18S sequences and protein-encoding mitochondrial sequences has been explored by sampling additional taxonomic diversity within the Ascaridoidea. Nuclear-encoded rRNA sequences, particularly the conservatively

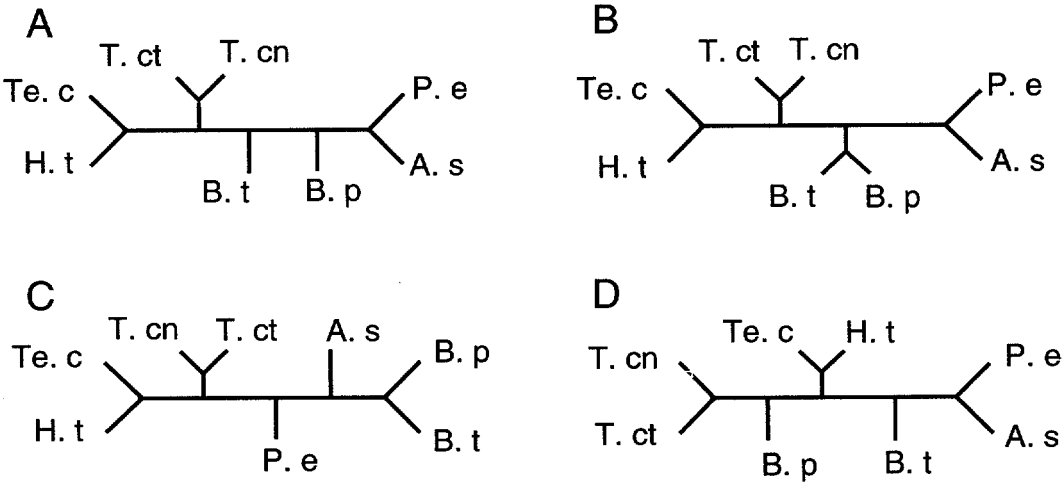

FIG. 3. Alternative tree topologies for 18S/28S data compared by statistical methods (12,18). A) Optimal maximum likelihood tree. B) Optimal parsimony tree, not significantly worse than Fig. 3A. C) Tree with traditional *Ascaris* plus *Baylisascaris* clade, not significantly worse than Fig. 3A. D) Tree with non-monophyletic Ascaridinae, significantly worse than Fig. 3A.

evolving 18S subunit, have traditionally been used to infer relationships among relatively distantly related organisms. The expectation for the Ascaridoidea is that 18S rRNA sequences should be informative for defining relationships among major lineages of ascaridoids, but remain relatively uninformative for closely related taxa (for example, species within a genus). In contrast, most mitochondrially encoded genes are expected to be characterized by more rapid rates of nucleotide substitution. Thus, cytochrome oxidase subunit II (COX II) sequences are expected to show more variation among closely related species and have greater utility for estimating relationships for closely related taxa. These expectations have been confirmed for the ascaridoid species compared (Fig. 4); on a per-nucleotide basis, COX II sequences have a 10-fold greater number of phylogenetically informative sites (parsimony criterion) than 18S sequences.

Full-length sequences of the 18S ribosomal RNA have been obtained for 13 species by using PCR-amplified DNA as the template for double-stranded cycle sequencing (23). In addition, internal oligonucleotide primers were used to amplify 90% of the mitochondrial COX II gene. For the following preliminary analyses, partial COX II sequence data (300 nucleotides) and full-length 18S rRNA sequences were combined for phylogenetic inference. For the 18S sequences, nucleotide alignments were performed using the CLUSTAL V computer program (9). For COX II, amino acid sequences were deduced using the nematode mitochondrial code (30), and the resulting polypeptides were aligned using CLUSTAL V. This alignment was used to infer positional homology for the COX II nucleotide sequences. Phylogenetically informative sites in the aligned sequences were identified and analyzed by MP using PAUP version 3.0 (26). For COX II, only informative sites corresponding to first and second positions of codons were included in the analysis; third position changes were excluded because these sites are likely to be homoplastic due to multiple nucleotide substitutions.

Inferred trees in this preliminary analysis were rooted by *H. tunicatus,* which was the basal ingroup taxon in previous analyses that included *C. elegans* as the outgroup (18). To compensate for potential differences in the rate of transition versus transversion substitutions in these data, MP analysis was employed using a stepmatrix that weighted transversions five times greater than transitions. The weighted MP analysis yielded a single shortest tree of 236 steps (Fig. 4); unweighted MP analysis of these data, including phylogenetically informative gaps in the 18S sequence, yielded the same tree topology (102 steps). Bootstrap MP support for clades in the weighted analysis exceeded 60% (Fig. 4) in groups including: (*A. suum, P. equorum*), the Ascaridinae (*Ascaris, Parascaris, Baylisascaris,* and *Toxascaris*), the Goeziinae sensu Gibson (*Iheringascaris, Hysterothylacium,* and *Goezia*), and the Anisakinae (*Anisakis, Pseudoterranova*). The topology of the minimal-length MP tree is generally consistent with traditional expectations at lower taxonomic levels. At higher taxonomic levels this tree topology is inconsistent with certain proposed arrangements (5,7), but consistent with others (3,24). For example, representative taxa from the Anisakidae, defined as the Anisakinae and Goeziinae sensu Gibson (5) or Anisakinae, Goeziinae, and Raphidascaridinae sensu Hartwich (7), are not monophyletic. By contrast, this tree topology is consistent with the taxonomy of Sprent (24), who places *Iheringascaris, Hysterothylacium,* and *Goezia* in one subfamily (Raphidascaridinae) and *Anisakis* and *Pseudoterranova* in another (Anisakinae). However, in contrast to the taxonomy of most authorities (3,5,7,24), the Ascarididae, which includes the Ascaridinae and Toxocarinae, is not monophyletic. An interesting feature of Figure 4 is the clade consisting of the Ascaridinae plus *T. caballeroi.* Although this grouping was unexpected based on current classifications and the results of previous analyses based on partial 18S and

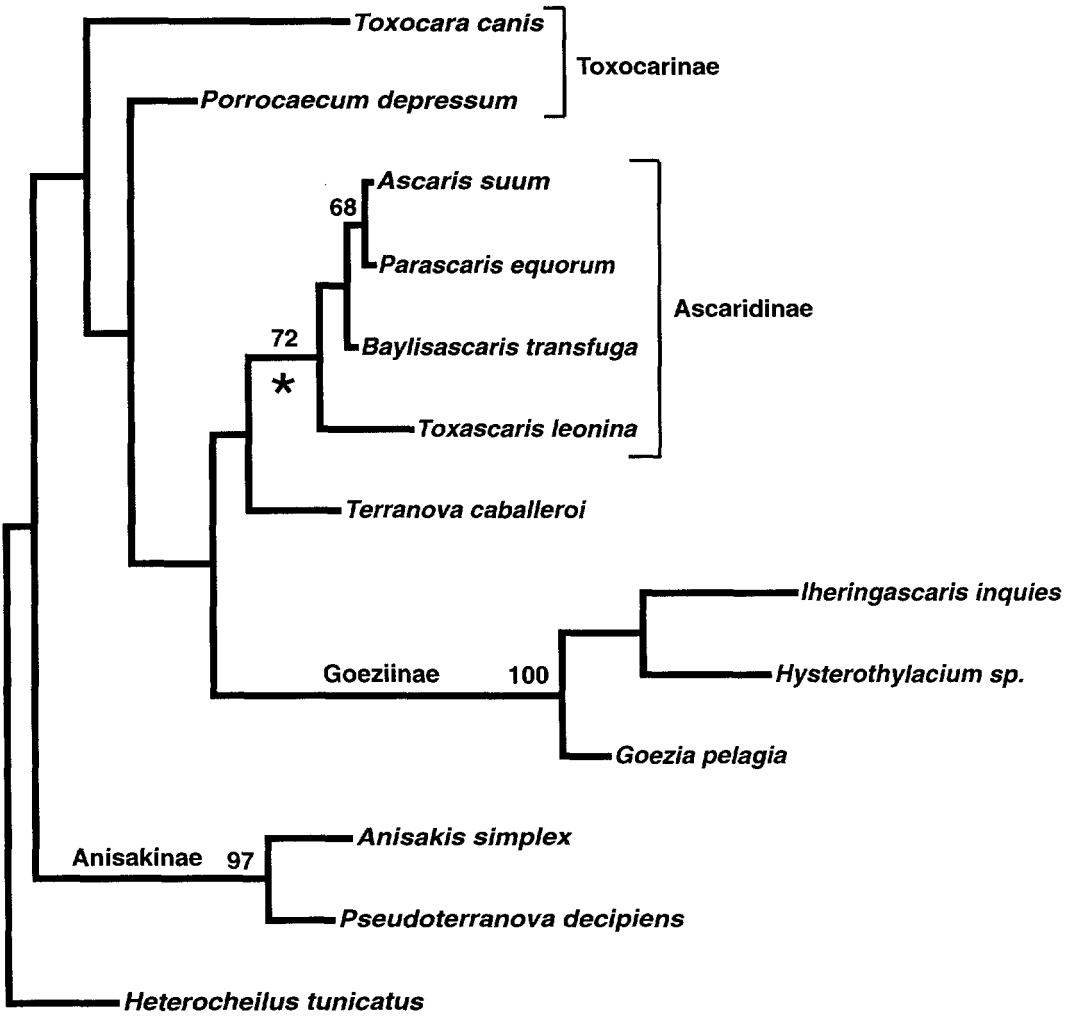

FIG. 4. Preliminary phylogenetic analysis based on transversion-weighted maximum parsimony analysis of data from full-length 18S rRNA and partial COX II (mitochondrial) sequences. Branch lengths are scaled to the number of substitutions inferred by parsimony analysis. Bootstrap percentages of clades are shown for groups found in more than 60% of the replicates. The asterisk marks the hypothesized loss of the ventriculus for the ingroup taxa based on the most parsimonious mapping of this character on the molecular tree. Sequence data (18S and partial COX II sequences with ambiguities) and alignments used for this analysis are available from the author; full-length 18S and complete sequences of this 629 bp COX II product will be deposited in GenBank and released with the publication of a more comprehensive analysis.

28S sequences (Fig. 2), recent morphological observations (3) suggest that the genus is not monophyletic and that *T. caballeroi* should not be considered closely related to the anisakids.

With the understanding that this phylogenetic hypothesis (Fig. 4) is preliminary, one potential use for the inferred tree is to assess possible patterns of evolution in morphological characters and life-cycle features of ascaridoids. Mapping morphological characters on an independently derived phylogenetic hypothesis can be useful for understanding which "key" taxonomic characters emphasized previously by systematists are homoplastic, provided that the inferred tree is reasonably accurate. For example, the presence or absence of the ventriculus among taxa has a consistency index of 1.0 on this tree because it can be explained most parsimoniously by a single loss (Fig. 4) in the com-

mon ancestor of the Ascaridinae sensu Gibson (5). By contrast, certain morphological features appear to be highly homoplastic as reflected by the distribution of required changes in character states on the tree. For example, consistency indices for the presence or absence of two structures—the intestinal caecum and cervical alae—are each 0.25. If this sequence-based hypothesis is an accurate representation of evolutionary history, then these results suggest that the similarity in certain complex structural features among ascaridoid taxa may result from convergent or parallel evolution. Obviously, a hypothesis concerning genealogical relationships that is focused exclusively on one or more of such features will be misleading. It should be noted that these few examples of homoplastic evolution do not establish a pattern for structural characters in ascaridoid species. A comparative analysis of the actual homoplastic content of morphological versus molecular characters is only possible with respect to the true phylogeny for ascaridoid nematodes. In the absence of a known history, the best estimate of phylogeny is likely to result from the combined analysis of all of the available high-quality data, including selected molecular, morphological, and life history characters.

Perspectives and Future Directions

Nucleotide sequence data have great potential utility for estimating phylogenetic relationships for organisms such as nematodes, in which morphological convergence and parallelism, or a lack of defined traditional characters, may hinder studies of evolutionary history. However, the youthful optimism once characteristic of the discipline of molecular systematics has been replaced by a more balanced perspective that recognizes the advantages and limitations of nucleotide sequence data for inferring organismal relationships. Rather than being a deterministic process ("phylogenetic reconstruction"), phylogenetic methods involve estimating an unknown (organismal history) and using tree-building procedures that require explicit or implicit assumptions and models that are inherently probabilistic. Trees inferred from sequence data should be viewed as provisional hypotheses that may change pending the acquisition of more data or as a result of applying improved models of inference. Given these limitations, assessing the reliability of a particular phylogenetic hypothesis should be regarded as part of the standard operating procedure for macroevolutionary studies. Improvements in the efficiency of nucleotide sequencing will be important for molecular systematics because documenting patterns of congruence among independent phylogenies inferred from several unlinked genes will become an increasingly important comparative approach. Finally, some of the most important advances in molecular systematics will involve determining the conditions under which current tree inference procedures may be misleading for data sets of realistic size, and developing new analytical approaches to overcome some of these difficulties.

Literature Cited

1. Avise, J. C. 1994. Molecular markers, natural history, and evolution. New York: Chapman and Hall.

2. Brooks, D. R., and D. A. McLennan. 1991. Phylogeny, ecology, and behavior. Chicago: University of Chicago Press.

3. Fagerholm, H.-P. 1991. Systematic implications of male caudal morphology in ascaridoid nematode parasites. Systematic Parasitology 19:215–228.

4. Felsenstein, J. 1988. Phylogenies from molecular sequences: Inference and reliability. Annual Review of Genetics 22:521–565.

5. Gibson, D. I. 1983. The systematics of ascaridoid nematodes—a current assessment. Pp. 321–338 *in* A. R. Stone, H. M. Platt, and L. F. Khalil, eds. Concepts in nematode systematics. New York: Academic Press.

6. Hafner, M. S., P. D. Sudman, F. X. Villablanca, T. A. Spradling, J. W. Demastes, and S. A. Nadler. 1994. Disparate rates of molecular evolution in cospeciating hosts and parasites. Science 265:1087–1090.

7. Hartwich, G. 1974. Keys to genera of the ascaridoidea. Pp. 1–15 *in* R. C. Anderson, A. G. Chabaud, and S. Wilmott, eds. CIH keys to the nematode parasites of vertebrates. Farnham Royal, England: Commonwealth Agricultural Bureaux.

8. Harvey, P. H., and M. D. Pagel. 1991. The com-

parative method in evolutionary biology. Oxford, England: Oxford University Press.

9. Higgins, D. E., A. J. Bleasby, and R. Fuchs. 1992. CLUSTAL V: Improved software for multiple sequence alignment. Computer Applications in the Biosciences 8:189–191.

10. Hillis, D. M., J. P. Huelsenbeck, and C. W. Cunningham. 1994. Application and accuracy of molecular phylogenies. Science 264:671–677.

11. Inglis, W. G. 1965. Patterns of evolution in parasitic nematodes. Pp. 79–124 *in* A. E. R. Taylor, ed. Evolution of parasites. Oxford, England: Blackwell Scientific Publications.

12. Kishino, H., and M. Hasegawa. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. Journal of Molecular Evolution 29:170–179.

13. Koop, B. F., and M. Goodman. 1988. Evolutionary and developmental aspects of two β-hemoglobin genes (ϵ^M and β^M) of opossum. Proceedings of the National Academy of Science USA 85:3893–3897.

14. Lanyon, S. M. 1988. The stochastic mode of molecular evolution: What consequences for systematic investigations? Auk 105:565–573.

15. Li, W.-H., M. Tanimura, and P. M. Sharp. 1988. Rates and dates of divergence between AIDS virus nucleotide sequences. Molecular Biology and Evolution 5:313–330.

16. Miyamoto, M. M., and J. Cracraft, eds. 1991. Phylogenetic analysis of DNA sequences. New York: Oxford University Press.

17. Nadler, S. A. 1987. Biochemical and immunological systematics of some ascaridoid nematodes: Genetic divergence between congeners. Journal of Parasitology 73:811–816.

18. Nadler, S. A. 1992. Phylogeny of some ascaridoid nematodes, inferred from comparison of 18S and 28S rRNA sequences. Molecular Biology and Evolution 9:932–944.

19. Nei, M. 1987. Molecular evolutionary genetics. New York: Columbia University Press.

20. Osche, G. 1963. Morphological, biological, and ecological considerations in the phylogeny of parasitic nematodes. Pp. 283–302 *in* E. C. Dougherty, ed. The lower metazoa, comparative biology and phylogeny. Berkeley, CA: University of California Press.

21. Penny, D., M. D. Hendy, and M. A. Steel. 1992. Progress with methods for constructing evolutionary trees. Trends in Ecology and Evolution 7: 73–79.

22. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of β-globin genomic sequence and restriction site analysis for diagnosis of sickle cell anemia. Science, 230:1350–1354.

23. Smith, D. P., E. M. Johnstone, S. P. Little, and H. M. Hsiung. 1990. Direct DNA sequencing of cDNA inserts from plaques using the linear polymerase chain reaction. Biotechniques 9:48–54.

24. Sprent, J. F. A. 1983. Observations on the systematics of Ascaridoid nematodes. Pp. 303–319 *in* A. R. Stone, H. M. Platt, and L. F. Khalil, eds. Concepts in nematode systematics. New York: Academic Press.

25. Sprent, J. F. A. 1992. Parasites lost? International Journal for Parasitology 22:139–151.

26. Swofford, D. L. 1989. PAUP user's manual. Version 3.0. Champaign, IL: Illinois Natural History Survey.

27. Swofford, D. L., and G. J. Olsen. 1990. Phylogeny reconstruction. Pp. 411–501 *in* D. M. Hillis, and C. Moritz, eds. Molecular Systematics. Sunderland, MA: Sinauer.

28. Tagle, D. A., B. F. Koop, M. Goodman, J. L. Slightom, D. L. Hess, and R. T. Jones. 1988. Embryonic ϵ and γ genes of a prosimian primate (*Galago crassicaudatus*): Nucleotide and amino acid sequences, developmental regulation, and phylogenetic footprints. Journal of Molecular Biology 203:439–455.

29. Wilson, A. C. 1985. The molecular basis of evolution. Scientific American 253:164–173.

30. Wolstenholme, D. R. 1992. Animal mitochondrial DNA: Structure and evolution. International Review of Cytology 141:173–216.