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Phylogenetic Relationships and Tempo of Early Diversification in *Anolis* Lizards

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Abstract.— We examine phylogenetic relationships among anoles using mitochondrial DNA sequences from the NADH dehydrogenase subunit 2 gene (*ND2*) and five transfer-RNA genes representing 1,455 alignable base positions and 866 phylogenetically informative characters (parsimony criterion). We also present 16 morphological characters for phylogenetic analysis. Our analyses yielded poorly-supported nodes deep in the anole tree but many well-supported nodes for more recent phylogenetic divergences. We test the hypothesis that the major clades of anoles form a hard polytomy and present a general statistical framework for testing hypotheses of simultaneous branching of lineages by using molecular sequence data. Our results suggest that rapid diversification early in the evolutionary history of anoles explains why numerous researchers have had difficulty reconstructing well-supported dichotomous phylogenetic trees for anoles. [*Anolis*; mitochondrial DNA; parametric bootstrap; permutation test; phylogeny; polytomy.]

Anoles are a classic example of adaptive radiation (Jackman et al., 1997), featuring occupation of a great variety of habitats and corresponding diversity in form (Williams, 1983; Losos et al., 1998). Nearly 400 species of anoles are recognized, 140 of which occur in the Caribbean (Williams, 1992; Powell et al., 1996); 111 of the Caribbean species occur on the Greater Antilles (Cuba, Hispaniola, Jamaica, and Puerto Rico). Most studies of evolutionary diversification in anoles have focused on the Greater Antilles, where endemic species occupy diverse ecological niches and as many as 11 species occur sympatrically (reviewed by Williams, 1983; Losos, 1994). Mainland anoles also exhibit extensive ecological differentiation (Pounds, 1988; Irschick et al., 1997). Despite the extensive study of these lizards, lack of a well-supported phylogenetic hypothesis has compromised evolutionary investigations.

Since Etheridge's (1959) osteological study, the first modern systematic treatment of the group, almost every newly available source of phylogenetic information has been applied to anoles, generating karyological (e.g., Gorman and Atkins, 1969; Gorman et al., 1969; see review by Williams, 1989), allozymic (Yang et al., 1974; Gorman and Kim, 1976; Gorman et al., 1980a, 1983; Burnell and Hedges, 1990; Hedges and Burnell, 1990), immunological (Gorman et al., 1980b; Wyles and Gorman, 1980a, 1980b; Shochat and Dessauer, 1981; Gorman et al., 1984; Hass et al., 1993), and DNA sequence (Hass et al., 1993) data. Nevertheless, a robust phylogenetic hypothesis relating the major groups of anoles has been elusive. Separate analyses of osteological, karyological, and immunological data collected through 1986 showed various points of agreement (Guyer and Savage, 1986); however, resolution in the osteological tree depended on successive weighting (Guyer and Savage, 1986), and a strict consensus of the unweighted osteological trees and the karyological and immunological trees was largely unresolved with regard to higher-level relationships (Fig. 1a; Cannatella and de Queiroz, 1989; see also Williams, 1989). A combined analysis of osteological, karyological, and electrophoretic data (Guyer and Savage, 1992) provided a resolved tree (Fig. 1b) only when the characters were successively weighted. A more recent analysis of higher-level anole relationships (Hass et al., 1993), based on immunological and DNA sequence data, reconstructed only very short branches deep in the tree in the immunological analysis and

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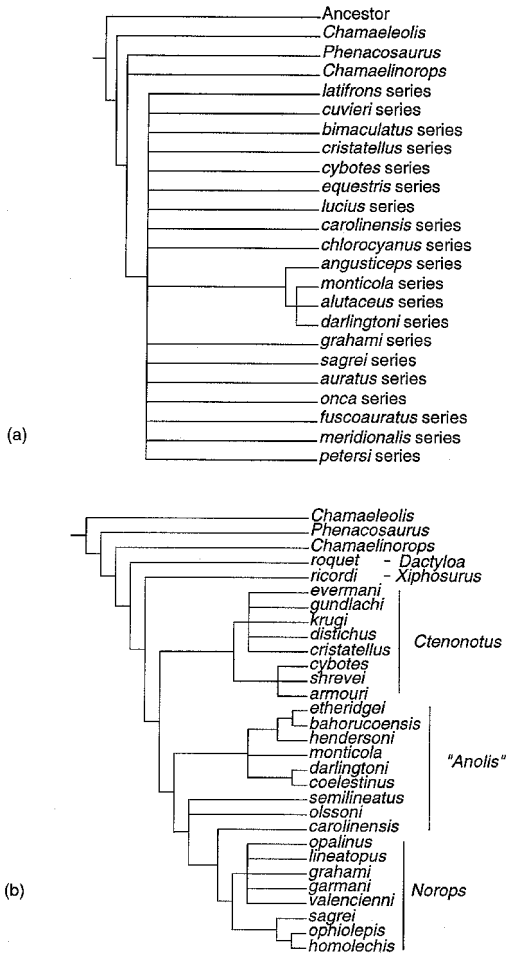

FIGURE 1. Previous phylogenetic hypotheses for anoles. (a) A strict consensus of osteological, allozymic, and karyological data from Cannatella and de Queiroz (1989). (b) Phylogenetic hypothesis of Guyer and Savage (1992), who divided traditional *Anolis* into five genera: *Anolis* (a subset of the genus as traditionally recognized), *Ctenonotus*, *Dactyloa*, *Norops*, and *Xiphosurus*. The placement of the most basal taxa is based solely on successively weighted osteological data. The rest of the analysis is based on a combination of allozymic, osteological, and karyological data.

provided very few well-supported branches in the DNA sequence analysis.

We present mitochondrial DNA sequences, representing the NADH dehydrogenase subunit 2 gene (*ND2*) and five transfer-RNA (tRNA) genes, and 16 morphological characters to test phylogenetic hypotheses for anoles. We present a statistical procedure for testing the hypothesis of simultaneous branching of multiple evolutionary lineages early in the evolution of anoles. Our results indicate that although simultaneous branching can be rejected for at least some of the lineages in question, it cannot be rejected for others. Patterns of rapid successive branching and simultaneous branching of lineages early in anole phylogenetic history may explain the inability of numerous studies to reconstruct a well-supported dichotomous phylogenetic tree.

PREVIOUS TAXONOMIC AND PHYLOGENETIC HYPOTHESES

Four genera are traditionally recognized among anoles (Williams, 1976, 1992): *Anolis*, containing > 300 species, and *Chamaeleolis*, *Chamaelinorops*, and *Phenacosaurus*, containing < 15 species combined. The relationships among these four genera are controversial. The phylogenetic placement of *Chamaelinorops*, a morphologically bizarre, monotypic Hispaniolan genus, has been particularly problematic. This genus is most notable for several derived characters—including interzygopophysial vertebral wings (Forsgaard, 1983), which are unlike anything displayed by other anoles and have few parallels in vertebrates. These data have been interpreted as indicating that *Chamaelinorops* is closely related to, but outside, the *Anolis* radiation (reviewed by Case and Williams, 1987), a hypothesis that has been supported by a parsimony analysis of Etheridge's (1959) osteological data (Guyer and Savage, 1986, 1992) and, to a limited extent, by an allozymic study of seven species (Case and Williams, 1987). Alternatively, immunological (Wyles and Gorman, 1980a) and DNA sequence (Hass et al., 1993) data have suggested that *Chamaelinorops* arose from within the Hispaniolan *Anolis* radiation, although Case and Williams (1987) interpreted some of these findings as an artifact of variation in rates of molecular evolution.

The Cuban genus *Chamaeleolis* has been placed outside of *Anolis* on the basis of morphological data (Guyer and Savage, 1986, 1992), but both immunological and DNA sequence data (Hass et al., 1993) suggest

that it falls within *Anolis*, close to the other West Indian giant anoles. Considering the morphological data, Etheridge (1959) and Etheridge and de Queiroz (1988) suggested that *Phenacosaurus* may be nested within *Anolis*, whereas Guyer and Savage (1986, 1992) placed *Phenacosaurus* outside of *Anolis*. No molecular data have been published previously for *Phenacosaurus*.

These contrasting interpretations of phylogenetic relationships have led to alternative scenarios concerning Caribbean faunal evolution. Williams (1969) postulated that Caribbean islands were originally populated by an early radiation of anoles, of which *Chamaelinorops* and *Chamaeleolis* are the only survivors, the rest having been supplanted by the later radiation of *Anolis*. In contrast, the immunological and DNA data suggest that *Chamaelinorops* and *Chamaeleolis* are part of the radiation of *Anolis*. Furthermore, rather than viewing the unique morphology of *Chamaelinorops* as the result of a long period of divergence, this latter interpretation suggests that rates of morphological evolution have varied substantially, with *Chamaelinorops* evolving rapidly relative to the other anoles.

Relationships within the large genus *Anolis* also have been controversial. Etheridge (1959) divided *Anolis* into two groups, termed the alpha and beta sections, each hypothesized to be monophyletic relative to the other (although not necessarily relative to the other anole genera). This traditional view, elaborated by Williams (1976), has been contradicted by immunological (Shochat and Dessauer, 1981; Gorman et al., 1984; Hass et al., 1993), allozymic (Burnell and Hedges, 1990), and DNA (Hass et al., 1993) studies and by a reevaluation of the osteological data (Guyer and Savage, 1986, 1992). These later studies suggest that the alpha section is paraphyletic, with the beta section arising from within it. Allozymic (Burnell and Hedges, 1990) and immunological (Gorman et al., 1984) data have also raised the possibility that the beta section is polyphyletic, with the West Indian betas being more closely related to the West Indian alphas than they are to the Central American betas.

On the basis of their successively weighted analyses of osteological and combined osteological, karyological, and electrophoretic data, Guyer and Savage (1986, 1992) proposed that the genus *Anolis* be split into five genera. Monophyly of the five proposed genera, however, is compatible only with the osteological analysis (but see Cannatella and de Queiroz, 1989). In the combined analysis of Guyer and Savage, (1992), *Anolis*—a subset of the genus as previously described—is not monophyletic. Hereafter, we use *Anolis* to refer to the genus as recognized by Etheridge (1959) and Williams (1969) unless otherwise indicated.

Tempo of Phylogenetic Diversification

Evolutionary theory suggests that in certain circumstances, such as after colonization of ecologically open territory, lineages should experience a burst of rapid evolutionary diversification (Simpson, 1953). If cladogenesis is truly simultaneous, the failure to resolve dichotomous relationships among the taxa in question is called a hard polytomy (Maddison, 1989). Multiple branching events that are extremely rapid in succession may be empirically indistinguishable from simultaneous branching. Several authors have inferred rapid diversification from molecular data. For example, the observation of very low and approximately equal amounts of molecular divergence among multiple species in a clade suggests the occurrence of simultaneous or rapid successive diversification in the recent evolutionary past (e.g., Brower, 1994). Alternatively, if the internal branches are much shorter than the terminal branches in a molecular phylogenetic analysis, rapid diversification in the more ancient evolutionary history of the group is suggested (Kraus and Miyamoto, 1991; Helm-Bychowski and Cracraft, 1993; Brown et al., 1994; Shaffer et al., 1997).

We concentrate here on testing hypotheses about the tempo of diversification. In particular, we examine the hypothesis that an observed polytomy is a hard polytomy, representing simultaneous or effectively simultaneous branching in the ancient his-

tory of a taxon, rather than a soft polytomy (Maddison, 1989), representing an analytical artifact. For example, if too few characters are used in an analysis, or if the characters used have undergone numerous, superimposed substitutions so that the molecule becomes uninformative for deep evolutionary divergences (substitutional saturation), a polytomy may be reconstructed and simultaneous branching may be inferred when the actual phylogenetic pattern was one of sequential branching (Fig. 2). One also might conclude erroneously that simultaneous branching occurred where sequential branching events obscure the fact that the temporal interval between the first and last branching events was relatively long (Fig. 3a).

We present a general strategy using several complementary statistical approaches for testing whether an observed polytomy is hard or soft (Fig. 4). In addition to examining the number and saturation of characters, we have developed tests utilizing subsampling of taxa which can help to distinguish sequential branching events from simultaneous or effectively simultaneous diversification (Fig. 3). If a long branch has been divided by many successive branching events, then appropriate subsets of species should reveal phylogenetic structure that is obscured when all species are analyzed together. In contrast, if simultaneous branching occurred, analyses using subsets of species should lack phylogenetic structure.

If the optimal trees, despite poor support of internal branches, accurately estimate the phylogenetic branching order of species, then the taxa most distant from each other on those trees are the ones most likely to reveal significant phylogenetic structure when analyzed as a subset. However, when phylogenetically randomized data (i.e., data meeting the expectation of simultaneous branching) are analyzed, a single most-parsimonious tree often results (Hillis and Huelsenbeck, 1992). Therefore, the appropriate null hypothesis for identifying phylogenetic structure is that a subset of species from a real data set does not contain branches that are better supported than those obtained from subsets of phylogenetically randomized data. We use this principle to test whether series of weakly supported nodes in the reconstructed phylogeny of anoles represent effectively simultaneous branching or an analytical artifact.

Materials and Methods

Sampling of Taxa

Based on morphological data, the closest out-groups for the anole clade are *Polychrus*, the leiosaurs, and the para-anoles (Etheridge and de Queiroz, 1988; Frost and Etheridge, 1989; Etheridge and Williams, 1991). We used *Polychrus acutirostris* and the leiosaur *Diplolaemus darwinii* as out-groups in this study; samples of 53 species of anoles constituted our in-group taxa. Sampling was biased toward Antillean taxa because most ecological studies and most of the taxonomic controversy regarding anoles have focused on the Caribbean.

Museum numbers for voucher specimens from which DNA was extracted (AMNH = the American Museum of Natural History, New York; IES = Instituto de Ecología y Sistemática, La Habana, Cuba; JBL = collection of Jonathan B. Losos; KdQ = Kevin de Queiroz, field series; LSU = Louisiana State University, Baton Rouge; MVZ = Museum of Vertebrate Zoology, University of California at Berkeley; USNM = United States National Museum of Natural History, Washington, DC) and GenBank accession numbers (in parentheses) in which DNA sequences are deposited are as follows: *Anolis acutus* JBL 871 (AF055927), *A. aeneus* JBL 442 (AF055950), *A. agassizi* LSU H5703 (AF055952), *A. ahli* USNM 497946 (AF055941), *A. aliniger* JBL AL2 (AF055960), *A. alutaceus* USNM 497976 (AF055972), *A. angusticeps* USNM 497987 (AF055968), *A. barahonae* JBL 1017 (AF055973), *A. bahorucoensis* USNM 314288 (AF055933), *A. bartschi* USNM 497997 (AF055961), *A. bimaculatus* USNM 321912 (AF055931), *A. brevirostris* USNM 314293 (AF055954), *A. carolinensis* JBL 671 (AF055969), *A. christophei* JBL 357 (AF055958), *A. coelestinus* USNM 314310 (AF055959), *A. cristatellus* JBL 259 (AF055928), *A. cuvieri* USNM 321864 (AF055974), *A. distichus* JBL 1021

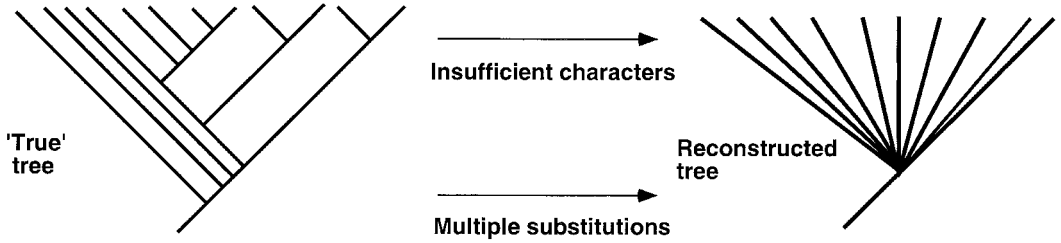

FIGURE 2. Analytical problems that can cause a lack of resolution. Insufficient sampling of characters results in few observed changes on internal branches. Substitutional saturation of the DNA sequences being compared will cause phylogenetically informative characters to be distributed randomly with respect to the phylogenetic topology, giving the false impression of simultaneous branching of lineages.

(AF055955), *A. equestris* JBL RB4 (AF055924), *A. etheridgei* USNM 314359 (AF055935), *A. garmani* JBL 22-26 (AF055937), *A. grahami* JBL 250 (AF055939), *A. humilis* MVZ FC14358 (AF055944), *A. insolitus* JBL 353 (AF055934), *A. krugi* USNM 321890 (AF055929), *A. limifrons* MVZ FC14143 (AF055943), *A. lineatopus* JBL 248 (AF055938), *A. lineatus* LSU H5450 (AF055936), *A. loysiana* USNM 498026 (AF055965), *A. luciae* USNM 321965 (AF055951), *A. lucius* USNM 498030 (AF055963), *A. luteogularis* USNM 498042 (AF055978), *A. marcanoi* JBL 274 (AF055956), *A. maynardi* JBL 400 (AF055970), *A. microtus* MVZ FC14156 (AF055947), *A. occultus* JBL 152 (AF055977), *A. olssoni* USNM 314397 (AF055945), *A. ophiolepis* USNM 498059

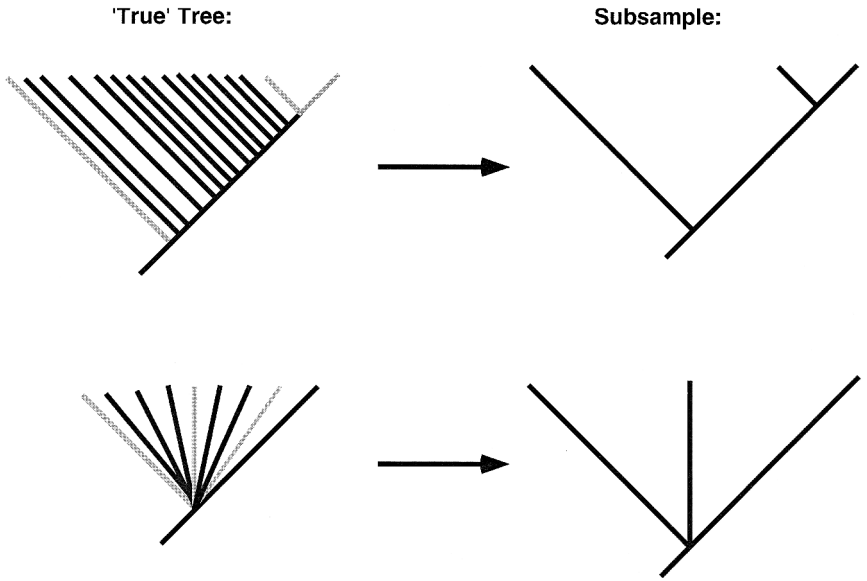

FIGURE 3. Detecting phylogenetic structure when relationships appear poorly supported on a tree containing all samples. Although the temporal interval between the first and last splitting events may be relatively long in a phylogeny that shows rapid sequential branching (top left), analytical problems associated with resolving short, internal branches may lead to the mistaken conclusion of hard polytomy. Subsampling of taxa (top right) permits detection of certain well-supported relationships, implying rapid but sequential dichotomous branching. Subsampling from a simultaneous branching of lineages (bottom left) should not yield well–supported relationships except by chance (bottom right).

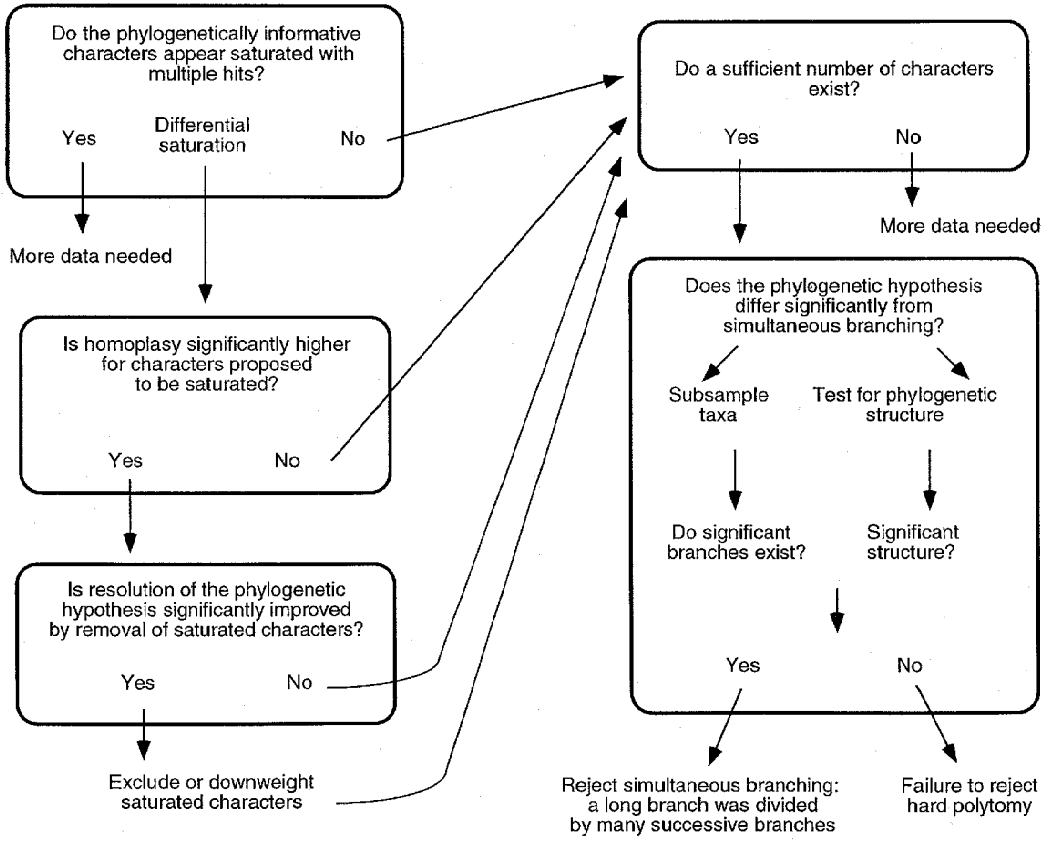

FIGURE 4. Analytical procedure recommended for using aligned molecular sequences to detect significantly supported relationships even when branches on the reconstructed tree appear poorly supported. Phylogenetically informative characters constitute sites at which derived states are shared among two or more in-group taxa in a multiple alignment of nucleic acid or protein sequences.

(AF055942), *A. paternus* KdQ 1769 (deposited at IES) (AF055966), *A. pumilis* USNM 498092 (AF055964), *A. richardi* JBL 439 (AF055949), *A. sagrei* JBL 407 (AF055940), *A. sheplani* USNM 314419 (AF055967), *A. strahmi* JBL 380 (AF055957), *A. stratulus* USNM 321908 (AF055930), *A. valencienni* JBL 262 (AF055953), *A. vanidicus* USNM 498115 (AF055971), *A. vermiculatus* USNM 498126 (AF055962), *A. wattsi* USNM 321758 (AF055932), *Chamaeleolis chamaeleonides* JBL 1016 (AF055976), *Chamaeleolis guamuhaya* KdQ 1681 (deposited at IES) (AF055975), *Chamaelinorops barbouri* JBL 365 (AF055946), *Diplolaemus darwinii* R. D. Sage 13041 (AF055925), *Phenacosaurus nicefori* J. Renjifo 2537 (AF055948), *Polychrus acutirostris* AMNH 10182 (AF055926).

Laboratory Protocols for DNA Sequence Data

Tissue samples were preserved in absolute ethanol and stored at –80°C. Genomic DNA was extracted from muscle or liver with use of the Qiagen QIAamp tissue kit. Amplification of genomic DNA was conducted for 30 cycles of denaturation at 94°C for 35 sec, annealing at 45–53°C for 35 sec, and extension at 70°C for 150 sec with 4 sec added to the extension per cycle. Negative controls were used in all amplifications to guard against contamination. Amplified products were purified on 1.5–2.5%

Nusieve GTG agarose gels and reamplified under similar conditions. Reamplified double-stranded products were purified on 2.5% acrylamide gels (Maniatis et al., 1982). Template DNA was eluted from acrylamide passively over 3 days, with replacement of the elution buffer (Maniatis et al., 1982) each day. Cycle-sequencing reactions were run with the Promega fmol DNA sequencing system for 30 cycles of denaturation at 95°C for 35 sec, annealing at 53–61°C for 35 sec, and extension at 70°C for 1 min per cycle. Sequencing reactions were run on Long Ranger™ sequencing gels for 4–12 hr at 38–42°C.

Amplification and sequencing primers (Fig. 5) are from Macey et al. (1997a, 1997b). DNA sequences encoding all of *ND2* and part of COI were aligned manually by using the PAUP text editor (Swofford, 1993). Alignments of sequences encoding tRNAs were constructed manually by comparison with secondary structural models (Kumazawa and Nishida, 1993; Macey and Verma, 1997). The origin for light-strand replication between the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes contains a 14–22 base-pair (bp) hypervariable region that was unalignable and therefore excluded from the analyses. The phylogenetic analyses were conducted with 1,455 alignable base positions.

Morphological Data

We scored 16 morphological characters (14 based on those of Etheridge [1959] and including all the variation scored by him) from museum specimens and Etheridge's data sheets. Four characters were ordered linearly, two characters were ordered by using a step matrix, and the remaining characters were treated as unordered. For the splenial character (#6), our step matrix implies that the splenial is more likely to be lost from a reduced state than from an unreduced one. For the inscriptional ribs (#13), losing or gaining a single rib or a connection costs one step, so that changes between states that differ by fewer ribs or connections are easier to obtain. This assumption is consistent with intraspecific variation in anole inscriptional ribs, which usually consists of states that differ from the mode by one rib or connection. A description of the characters and character states, as well as the step matrices used for partially ordered characters, can be found in Appendix 1.

Pairwise Comparisons of DNA Sequences

The number of pairwise differences between DNA sequences was calculated by examining each position separately in PAUP. To examine different categories of substitutions independently, we recoded the data to

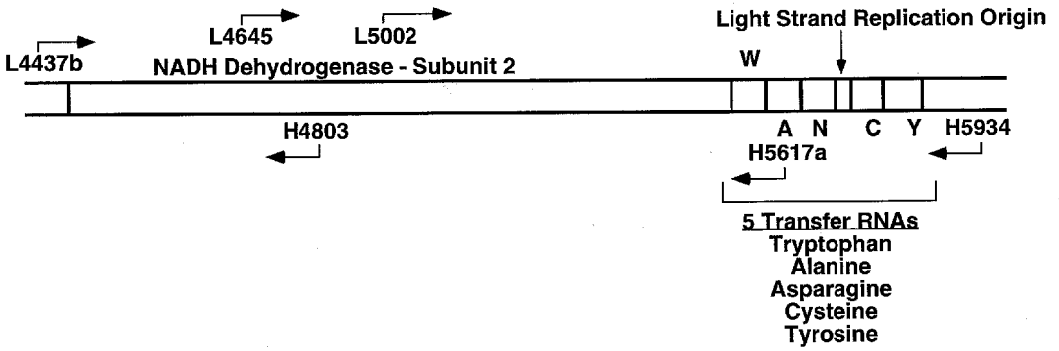

FIGURE 5. Primers used to amplify and sequence a total of 1455 bp pairs from the mitochondrial genes encoding NADH dehydrogenase subunit 2 and five RNAs of anoles. Primers H5617a, H5934, L4645, and L5002 are from Macey et al. (1997a). Primer L4437b is from Macey et al. (1997b). Primer H4803 is 5′-ACTTCTGGTAGTCAGAARTG-3′ (Macey, pers. comm.), where R denotes A or G bases. All primers were used for both amplification and sequencing.

score C and T differences, G and A differences, and transversional differences. Character states other than those of interest in a particular analysis were coded as missing and therefore were not included in counts of differences. The pairwise, first-position C to T differences in leucine codons were tallied by recoding CTN (N = G, A, T, or C) as CNN, and TTR (R = A or G) as TNN; all other sites were coded as missing. The number of silent transitions was calculated by adding third-position G/A and C/T differences plus first-position leucine C/T differences. The total of all categories of differences summed to equal the total number of differences as calculated by PAUP. For phylogenetic analyses that excluded silent transitions, we recoded the data such that third-position transitions were coded as purines (R) or pyrimidines (Y) and first-position leucine pyrimidines were coded as Y.

Maximum-likelihood distances were calculated by using the DNA distance program in PHYLIP (Felsenstein, 1993). The maximum-likelihood distances consider the base composition, differences in substitution rate among codon positions, and transition biases (Felsenstein, 1993). The values used for rate parameters were 2.5:1:7.1:1.5 for rates of changes at the 1st, 2nd, and 3rd codon positions and tRNA, respectively, and a 4.8:1 transition:transversion ratio; these values are based on pairwise comparisons within anoles for sequences differing by $< 5\%$.

We used PAUP* (version 4.59b, provided by David Swofford) to estimate the parameters alpha (the shape parameter of the gamma distribution, estimated by using four categories of change) (0.41), kappa (3.78), and the transition:transversion ratio (1.73); and to determine the empirical base frequencies. We implemented the parametric bootstrap analysis by using these values with the Siminator program (Huelsenbeck et al., 1996).

Estimation of Phylogenetic Trees

Phylogenetic trees were estimated by using the parsimony method as implemented in PAUP (Swofford, 1993) with 100 heuristic searches involving random-addition sequences and tree-bisection-and-reconnection (TBR) branch swapping. Bootstrap resampling was applied to assess support for individual nodes through use of 200 bootstrap replicates with simple addition and TBR. Neighbor-joining and Kitch analyses were performed by using PHYLIP 3.5 (Felsenstein, 1993).

The decay index (= branch support of Bremer, 1994) was calculated with the program Autodecay (Eriksson and Wikström, 1995), which creates a PAUP command file that makes a constraint tree for every node in the most-parsimonious tree and employs the reverse-constraints option of PAUP. Heuristic searches with random addition sequences (25 replicates) and TBR were used to find the shortest trees that lacked particular branches present in the most-parsimonious tree for the data set.

TABLE 1. Significance values for the four-species test of Felsenstein (1985b) for 80–157 characters. The decay index is equivalent to Felsenstein's statistic *S* when the two shortest trees are being compared.

No. of informative characters	Significant decay index
80–97	12
98–114	13
115	14
116	13
117–135	14
136–157	15

MacClade 3.0 (Maddison and Maddison, 1992) was used to randomize data through the "shuffle" option for statistical tests that utilize subsampling of taxa, to examine character changes on the most-parsimonious trees for contingency χ^2 tests of homoplasy (described below), and to constrain and examine 10,000 randomly generated trees for skewness tests (Hillis and Huelsenbeck, 1992). StatView (1994; Abacus Concepts, Berkeley, CA) was used to determine skewness of the lengths of the 10,000 randomly generated trees used in the skewness tests. To test for significant phylogenetic structure in subsamples containing four taxa, and assuming a molecular clock, we extended Table 2 of Felsenstein (1985b) to 157 characters, based on his formula 2 (Table 1 in the present report).

Tests for Substitutional Saturation among DNA Sequences

To test for saturation of different categories of substitutions, we used both a priori and a posteriori tests. For the a priori tests, the numbers of pairwise differences between the sequences were plotted against the maximum-likelihood estimates of pairwise divergence. Next, the numbers of silent transitions, tRNA stem, tRNA loop, first, second, and third codon-position transversions, and first- and second-position replacement transitions were plotted against corrected divergence. A second a priori method for detecting substitutional saturation involved using formula 11 from Holmquist (1983; see also Larson, 1991) to compare transition:transversion ratios observed for pairwise comparisons of sequences with the values expected for sequences having complete substitutional saturation.

For the a posteriori tests of whether the silent sites differed significantly from other kinds of change in the amount and distribution of homoplasy, we used a contingency χ^2 test (de Queiroz, 1989; Larson, 1994). The most-parsimonious tree derived from analysis of our molecular data was used to assess the numbers of homoplastic changes for silent transitions and for all other categories of change mentioned above.

Tests for Hard versus Soft Polytomies

We determined the support for branches (measured by using bootstrap values and decay indices; see below) on trees obtained from phylogenetically randomized data by using permutation testing (Archie, 1989) and the parametric bootstrap method (Huelsenbeck et al., 1996). We used these methods to establish a null distribution for examining the phylogenetic structure present in real data. The permutation test is similar to the a posteriori T-PTP test (Faith, 1991). However, subsampling species based on the phylogenetically randomized data makes this test more conservative than the original test.

We also employed Felsenstein's (1985b) test for significant branch support, using all subsamples containing three in-group taxa and one out-group. The value S (Felsenstein, 1985b), when calculated as the difference in tree length between the most-parsimonious tree and the next most-parsimonious tree, is equivalent to the decay index. Significance values for decay indices, calculated by assuming a molecular clock, were used to determine whether a particular subset of taxa contained significant phylogenetic structure (see Felsenstein, 1985b). The assumption of a molecular clock makes this test more powerful but less conservative (Felsenstein, 1985b).

Additional statistical criteria for evaluating polytomies were provided by tests for phylogenetic structure (Faith and Cranston, 1991; Hillis, 1991). The distribution of informative characters on a hard polytomy should be random with respect to the species. Results showing insignificant phylogenetic structure (Faith and Cranston, 1991; Hillis, 1991; Hillis and Huelsenbeck, 1992) have been used to demonstrate substitutional saturation (Hillis, 1991; Hillis and Huelsenbeck, 1992) or lack of adequate variation for phylogenetic analysis (Faith and Cranston, 1991). We employed these tests for phylogenetic structure to determine whether the early diversification of anoles could be distinguished statistically from simultaneous branching of lineages.

RESULTS

The aligned DNA sequences contained 1,037 variable and 866 phylogenetically informative characters (parsimony criterion) when including the out-group, and 1,016 variable and 854 informative characters within anoles (Fig. 6). If silent transitions were excluded, there were 772 informative characters among all sequences and 753 within anoles. An analysis restricted to including only amino-acid substitutions and transversions in tRNA genes identified 623 informative characters between all sequences and 611 within anoles. Within anoles, sequence divergence (uncorrected) ranged from 4% to 29%. Values between the in-group and out-group ranged from 25% to 32% (mean = 28%). Amino-acid divergence (uncorrected) averaged 15% between the in-

group and out-group and ranged from 0% to 15% within anoles. Scores for the morphological characters for the same taxa are given in Appendix 2.

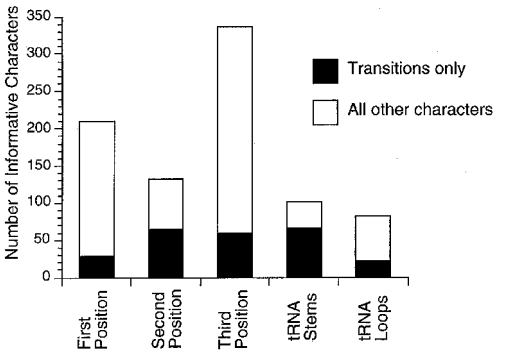

FIGURE 6. Numbers of informative anole characters categorized by position in a protein-coding sequence (first, second, or third codon position) or tRNA-coding sequence (corresponding to stems vs. loops in the functional tRNA) and type of substitution. All other characters = transversions alone or transversions in combination with transitions.

The Most-Parsimonious Tree

Parsimony analysis for all molecular data from this study produced a single most-parsimonious tree. Figure 7 shows this tree with bootstrap values above the nodes (Felsenstein, 1985a) and decay index values (Bremer, 1994) below the nodes. The length of the tree is 8,889 steps; the consistency index is 0.38. The removal of silent transitions significantly affected the tree only by improving support for the nodes indicated by plus symbols.

Numbers of Characters and Tests for Substitutional Saturation

Because our molecular data contained a large number of informative characters both within anoles and between the in-group and out-group, a simple lack of characters cannot explain the lack of well-supported branches deep in the tree (see below). The number of phylogenetically informative characters (866) was more than 15 times the number of taxa (55) sampled. Furthermore, phylogenetically informative variation in at least 100 characters was found for each of the deep internal branches that were examined with the four-taxon subsamples (results presented below). Our results also indicate that this lack of resolution cannot be attributed to substitutional saturation.

A priori tests.—Plotting maximum-likelihood distances versus observed differences between paired sequences is a heuristic test for saturation. Because the points are not independent, standard regression statistics cannot be applied. This analysis nonetheless can be a sensitive indicator of substitutional saturation when it occurs. Figure 8 shows a plot of the uncorrected numbers of differences between sequences versus maximum-likelihood distance. Substitutional saturation is inferred for plots showing a plateau rather than a linear increase in the number of uncorrected differences with maximum-likelihood distance. Only silent transitions showed the pattern expected for substitutional saturation. The other classes of characters accumulated differences in a linear fashion with different slopes. Separate plots of first-, second-, and third-position transversions as well as first- and second-position transitions were all linear, and these categories were combined for phylogenetic analysis. Table 2 shows the transition:transversion ratios at different sites and the expected ratios under complete saturation. Transition:transversion ratios for our aligned DNA sequences were 2–4 times the values expected for complete saturation according to equation 11 of Holmquist (1983).

TABLE 2. Transition:transversion ratios at different sites and the expected ratio with complete saturation of substitutions (Holmquist, 1983).

	Codon Position			
	1	2	3	tRNA
Observed	1.3	1.7	1.1	2.3
Expected	0.45	0.80	0.30	0.48

A posteriori tests for saturation.—A contingency χ^2 test for differential levels of homoplasy among categories of character change showed that silent transitions were significantly more homoplastic than all other

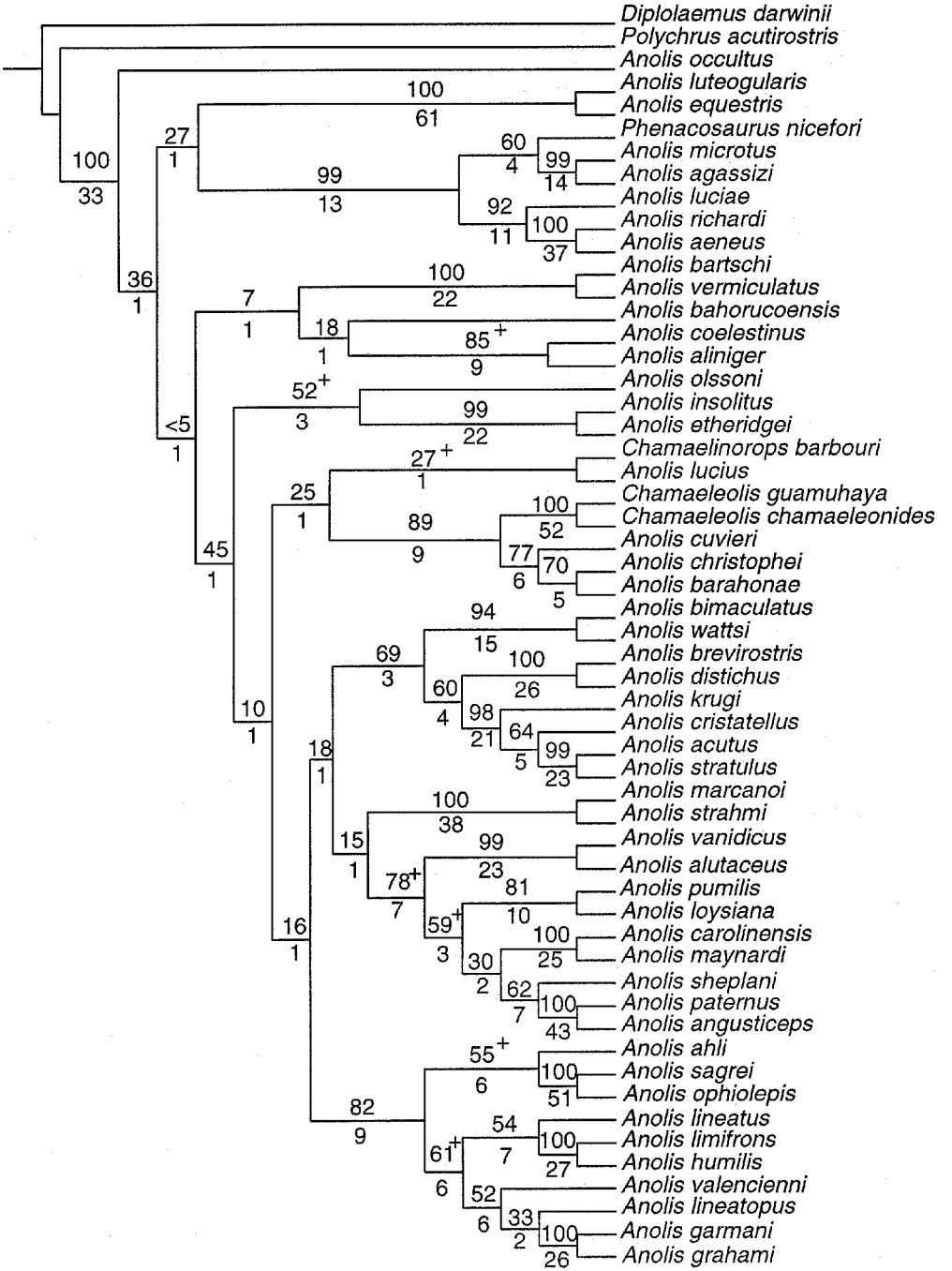

FIGURE 7. The most-parsimonious tree for anoles obtained with the molecular characters. Bootstrap values (%) are shown above and decay index values below the branches. The length of the tree is 8,889 steps, based on 1,037 variable characters, 866 of which are phylogenetically informative. The consistency index is 0.20 and the retention index is 0.35. + = nodes for which support increases when the analysis is repeated after omitting silent transitions.

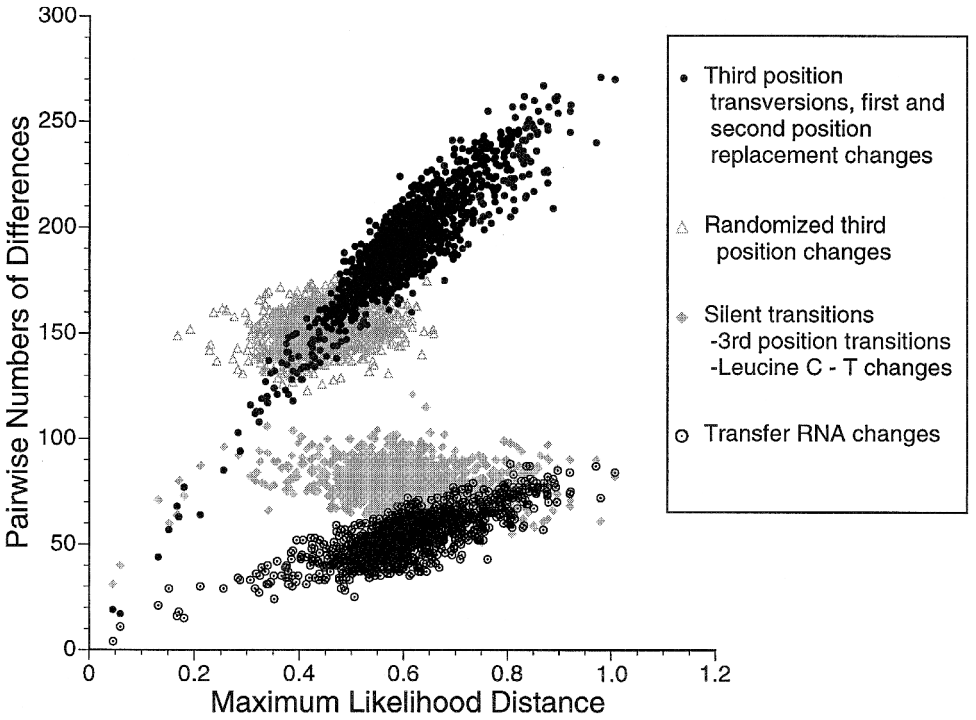

FIGURE 8. Heuristic tests for substitutional saturation. Numbers of bases differing among all pairs of aligned sequences are plotted against maximum-likelihood distances that correct for multiple substitutions occurring among the same paired sequences. Silent transitions include all transitions at third codon positions and C/T differences at the first position in leucine codons. First-position transitions (excluding leucine C/T changes), second-position transitions, and transversions at all three codon positions were combined into a single category because all had a linear relationship with maximum-likelihood distance. Phylogenetically randomized data for third codon positions of protein-coding genes show the expected relationship for saturated characters (slope $\approx$ 0). A positive linear plot suggests that substitutional saturation is not present, whereas a horizontal line or plateau at higher numbers indicates substitutional saturation. Substitutional saturation is indicated only for silent transitions at maximum-likelihood distance > 0.2. Third codon positions were randomized with respect to species, and the maximum-likelihood distances were recalculated to show the expected relationship for saturated characters.

changes (Table 3). We removed silent transitions and repeated the phylogenetic analysis to ask whether nodal support (as measured by bootstrap or decay index values, or both) was improved. Removal of silent transitions improved support significantly for only 7 branches (Fig. 7), including only 2 of the 15 deep branches in question, and slightly decreased support for the branches grouping closely related taxa for which silent transitions were not saturated. Although silent transitions were saturated for branches located deep in the tree, lack of support for deep branches appeared not to be an artifact of this saturation.

Analysis of Molecular and Morphological Data

An analysis of the 16 morphological characters (Appendix 2) alone revealed relatively weak support for all nodes in the tree (decay index – $\leq$3 within anoles), the most-parsimonious trees (Fig. 9) being 88 steps long. Although the 16 morphological characters did not by themselves yield a strongly supported phylogeny of anoles, they might contribute useful information when analyzed in combination with molecular data.

The combined analysis contained 881 informative characters for 55 species. The combined analysis provided a phylogenetic

TABLE 3. Comparison of levels of homoplasy occurring among silent transitions versus all transversions plus replacement transitions and transitions in tRNA genes. Character-state changes were tallied for all informative first and second codon positions, tRNA positions, and 100 randomly selected third codon positions. Character-state changes were categorized according to the pair of bases involved in the substitution. Different categories of change were tallied separately at each site examined for the contingency χ^2 test. The number of homoplasies equalled the number of changes for each category of change at each site that was in excess of the minimum number required for a single origin of each derived state. Homoplasy was measured by using the tree in Figure 7. Ambiguous changes were not counted.

	No. of homoplasies									
	0	1	2	3	4	5	6	7	8+	Total
Silent transitions	8 (20)[a]	20 (33)	18 (21)	22 (17)	13 (10)	10 (8)	8 (5)	7 (4)	18 (6)	124
All other changes	72 (60)	111 (98)	66 (63)	45 (50)	28 (31)	21 (23)	10 (13)	7 (10)	6 (18)	366
Total	80	131	84	67	41	31	18	14	24	490

[a]Expected values are in parentheses.
$\chi^2 = 60.6, p < 0.001$.

hypothesis (Fig. 10) that was largely congruent with the analysis of DNA sequences alone, differing only for some groups in which relationships were poorly resolved. Decay indices were improved for seven deep branches in the tree.

The mitochondrial 16S DNA sequence data from Hass et al. (1993) and the allozymic data from Burnell and Hedges (1990) were analyzed together with the morphological and molecular data presented here for 19 species common to these

TABLE 4. Hypotheses tested by using the Wilcoxon signed-ranks test with the molecular data. The null hypothesis is that the shortest tree(s) corresponding to the conditions listed below are not significantly different in length from the most-parsimonious tree. A significant result indicates that the conditions listed below require trees that are significantly longer that the most-parsimonious tree.

Hypothesis	n[a]	Z[b]	P
A. *Anolis* is monophyletic	234	3.78	< 0.001
B. Monophyly of			
Ctenonotus	14	0.8	n.s.
Dactyloa	33	0.73	n.s.
Xiphosurus	38	0.92	n.s.
Anolis (sensu Guyer and Savage, 1992)	263	3.8	< 0.001
C. Nonmonophyly of			
Norops (beta section)	246	0.40	n.s.
D. Species on each island in the Greater Antilles form a monophyletic radiation	344	5.7	< 0.001
E. Monophyly of			
Cuba	287	4.1	< 0.001
Hispaniola	331	4.0	< 0.001
Puerto Rico	221	2.92	< 0.004
F. Nonmonophyly of			
Jamaica	200	0.19	n.s.

[a]Number of characters that differed in minimum numbers of changes on the two trees.
[b]Normal approximation (Zar, 1984).
n.s., not significant.

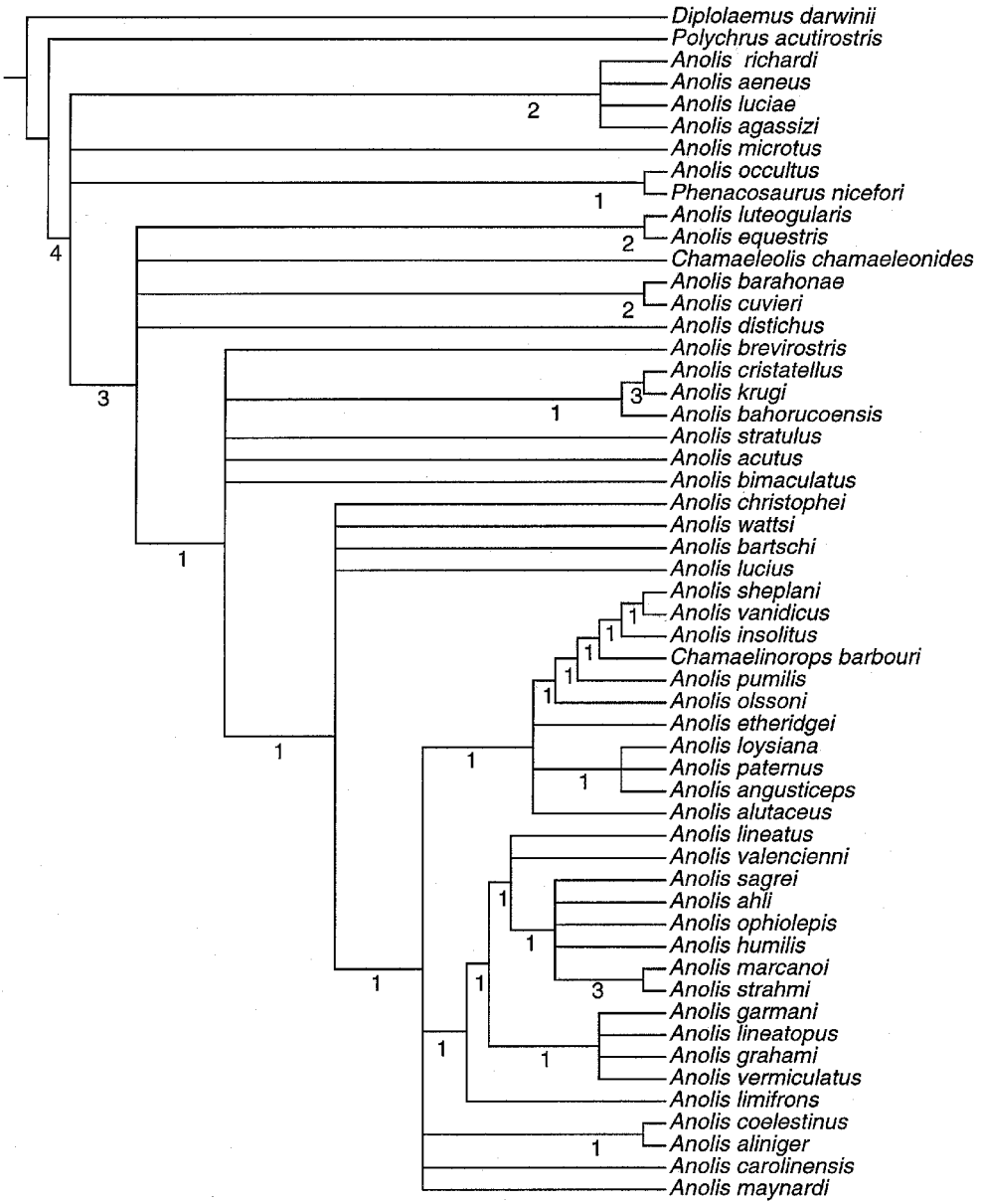

FIGURE 9. A strict consensus of 15,296 equally most-parsimonious trees generated from the 15 morphological characters (Appendix 2). Decay indices are shown below branches. The length of the tree is 88 steps. Morphological data are not available for *Chamaeleolis guamuhaya*.

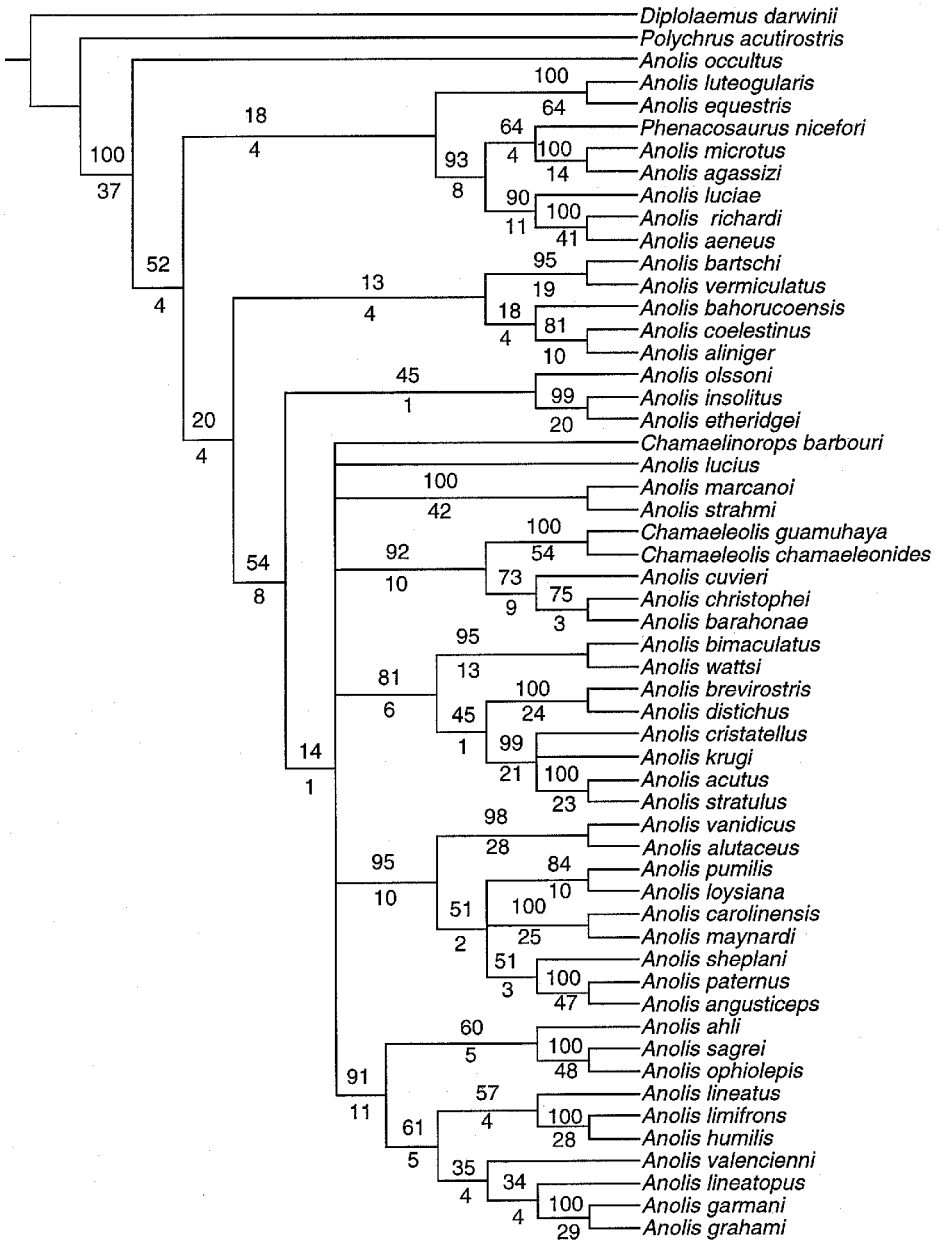

FIGURE 10. A strict consensus of the four equally most-parsimonious trees, based on a combined analysis of our molecular and morphological data. The consistency index of all four trees is 0.21 and the retention index is 0.35. Bootstrap values (%) are shown above and decay index values below the branches. The length of the tree is 9,020 steps, based on 881 informative characters.

different studies. The most-parsimonious tree from this analysis (not shown) preserved the well–supported branches of the trees presented above (Figs. 7, 10) and contained conflicts only for branches that were poorly supported in both analyses. As in our earlier analyses, *A. occultus*, the *A. latifrons* series (represented by *A. luciae*), and *A. equestris* occupy basal positions on this tree; *Chamaeleolis* and *Chamaelinorops* are both nested within *Anolis*.

Tests of Previous Phylogenetic Hypotheses

Using the Wilcoxon signed-ranks test (Templeton, 1983; Larson, 1994) with our molecular data (Table 4), we tested the monophyly of various previously proposed taxa, the hypothesis that the Caribbean anole fauna constitutes a monophyletic group, and the possibility that endemic species of Cuba, Hispaniola, and Puerto Rico form monophyletic groups for each site (Table 4). Our data rejected the hypothesis of monophyly of *Anolis* as traditionally conceived, as well as *Anolis* in the sense of Guyer and Savage (1986, 1992). Among the Caribbean Islands, only Jamaican anoles (not including *A. sagrei*, which only recently colonized Jamaica [Williams, 1969]) can be considered a monophyletic group.

Testing the Null Hypothesis of Simultaneous Branching of Lineages

Reconstructions of branch lengths.—Parsimony reconstructions of the numbers of unambiguous changes on the branches of the tree showed that deep internal branches (shown in bold on Fig. 11) were relatively short. Branch lengths calculated by using the neighbor-joining method (Saitou and Nei, 1987) and maximum-likelihood distances showed even shorter internal branches (Fig. 12a). Maximum-likelihood distances partitioned on the most-parsimonious trees (assuming a molecular clock) also produced short branches deep in the tree (Fig. 12b), six of which had a length of zero. The same pattern was evident for the 21 equally most-parsimonious trees based on amino-acid changes and tRNA transversions (not shown); deep branches in the tree were relatively short.

Branches having < 70% bootstrap values (Hillis and Bull, 1993) and decay indices < 5 (shown in bold on Fig. 11), even after silent transitions were removed, were tested against the hypothesis of a hard polytomy. By this criterion, the null hypothesis is that 17 taxa (clades and individual species marked in Fig. 11) originated in a simultaneous branching of lineages early in the phylogenetic history of anoles.

Permutation and parametric-bootstrap tests.—The permutation analysis and parametric bootstrap simulations of simultaneous branching were performed for 17 species (representing the 17 clades connected by short internal branches) and 2 out-groups. For the permutation analysis, states were randomized among the 17 species for informative DNA characters (with silent transitions removed) to produce 100 data matrices. The two out-group sequences were not included in these randomizations because they were not part of the hypothesis of simultaneous branching of lineages. Randomizations created the expected pattern of character distribution for simultaneous branching (Archie, 1989). Each randomized data set (plus out-group species *Diplolaemus darwinii* and *Polychrus acutirostris*) was analyzed with PAUP. Of the 100 randomizations, 39 resulted in a single most-parsimonious tree, and all but 1 randomization involved fewer than eight equally most-parsimonious trees.

From each randomized data set we took two subsamples of four taxa based on the most-parsimonious tree, or based on a majority-rule consensus of the equally most-parsimonious trees (Fig. 13). All subsamples contained four in-group species and two out-group species. Using the tree derived from randomized data, we selected the first subsample to maximize the number of nodes separating two species that represented a clade nested high in the tree from their two most distant relatives among the in-group species being examined (Fig. 13a). The branch of interest in this subsample is the one immediately ancestral to the nested clade. The second subsample was selected to maximize the number of nodes occurring between three species representing a nested

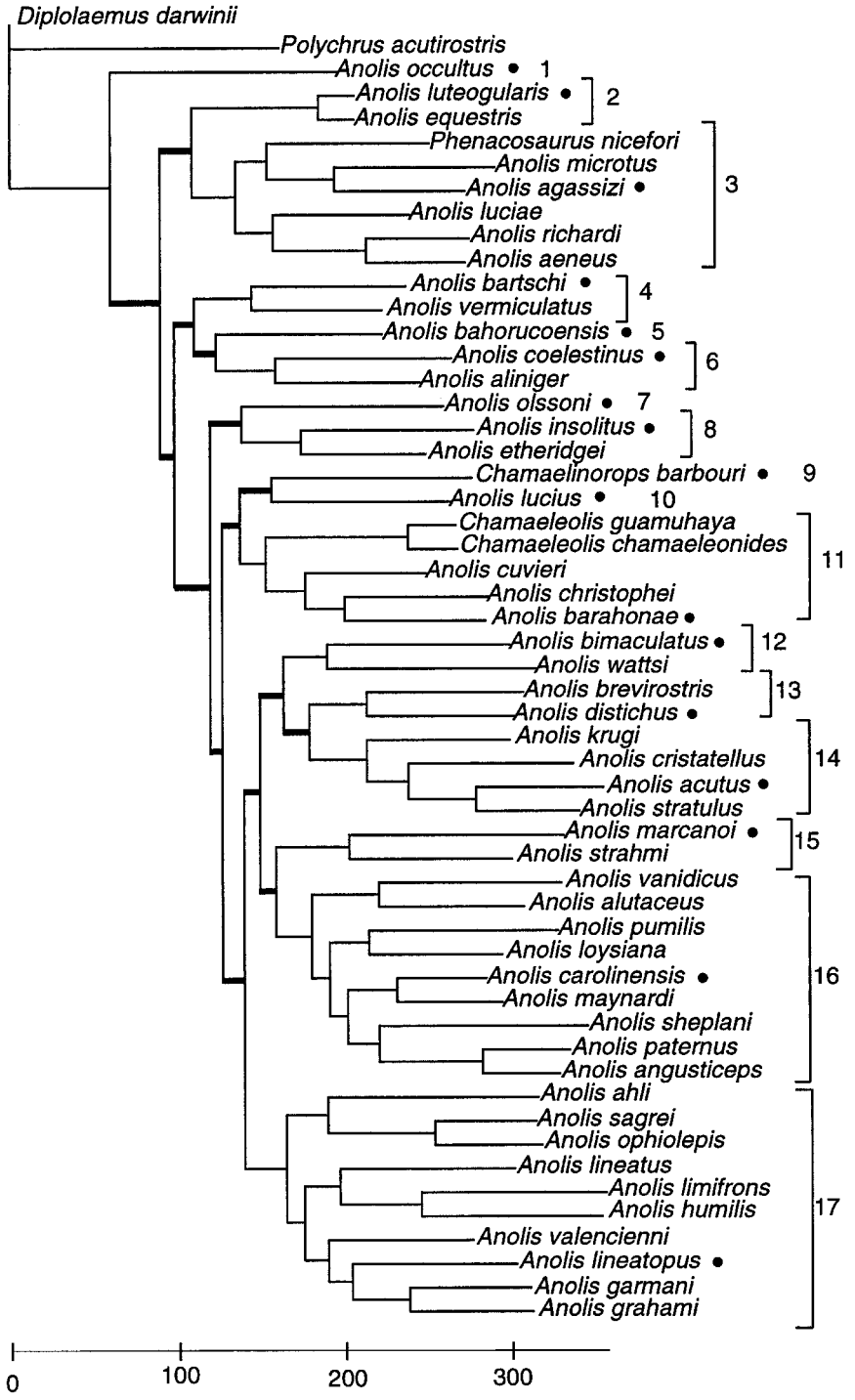

FIGURE 11. The 17 anole clades whose relationships were tested with subsampling tests; species indicated with a dot were used in the tests. Branch lengths are proportional to the numbers of unambiguous changes inferred by using maximum parsimony.

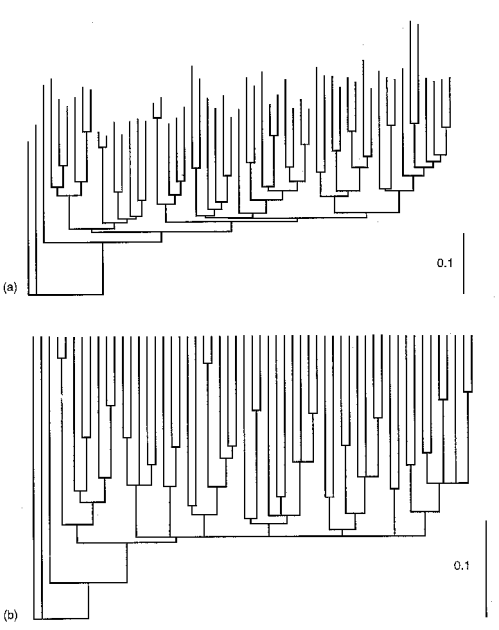

FIGURE 12. Reconstruction of anole branch lengths based on the molecular data. Calibrations show the length of a branch corresponding to a maximum-likelihood distance of 0.1. (a) Neighbor-joining tree based on maximum-likelihood distances. (b) Maximum-parsimony topology with distances reconstructed by using the Kitch method (assuming a molecular clock) and maximum-likelihood distances.

clade and their most distant relative within the in-group on a tree derived from randomized data (Fig. 13b). The branch of interest in the second subsample is again the one immediately ancestral to the nested clade. Choices between taxa equivalent by these criteria were made randomly (Fig. 13). If a majority-rule consensus precluded use of these criteria (only two cases), then one tree chosen randomly from the equally most-parsimonious trees was used for subsampling taxa.

We obtained bootstrap and decay index values for the branches of interest, using *Diplolaemus* and *Polychrus* as out-groups in phylogenetic analyses of these subsamples for the randomized data. Values corresponding to the 95th percentile were used as critical values for significance tests (Fig. 14). The critical values were found to be as follows: a decay index of 13 (under each subsampling regime, and combined) and bootstrap values of 94% (subsampling criterion 1), 97% (subsampling criterion 2), and 95% (combined) (Fig. 14). These results were obtained by using rooted trees; for a comparable analysis using trees unrooted for the four in-group taxa, the critical values were 96% for the bootstrap value and 17 for the decay index. Because the bootstrap values obtained in this analysis are close to the maximum possible value (100%), only the decay indices, which are unbounded, were used for subsequent analyses.

For the parametric bootstrap, maximum-likelihood estimates of branch lengths were made by using the topology for 17 subsampled in-group taxa and 2 out-groups that was consistent with the most-parsimonious tree based on all taxa. Maximum likelihood then was used to estimate transition:transversion ratios and the gamma distribution of rate variation from the same topology. All nodes connecting the 17 species were set to zero, making a simultaneous branching of 17 lineages, and 100 simulated data sets were created. For each simulated data set, all possible sets of four species were examined and the decay index was determined for the single internal branch that connected the four species. The distribution of all possible decay indices for the 100 randomized data sets (238,000 decay indices) was used as the null distribution for subsamples of four from the actual data. The critical value for the decay index was 13.

Our permutation analysis produced more–conservative criteria for significance than did our parametric bootstrap, as shown when comparing unrooted trees for the four in-group species. Two factors potentially contributed to this difference. First, our permutation analysis was performed on a data set from which the silent transition substitutions had been removed, whereas these characters were included in our parametric-bootstrap analysis. Second, the permutation test was designed to sample only the highest possible decay indices for each randomized data set, whereas the parametric

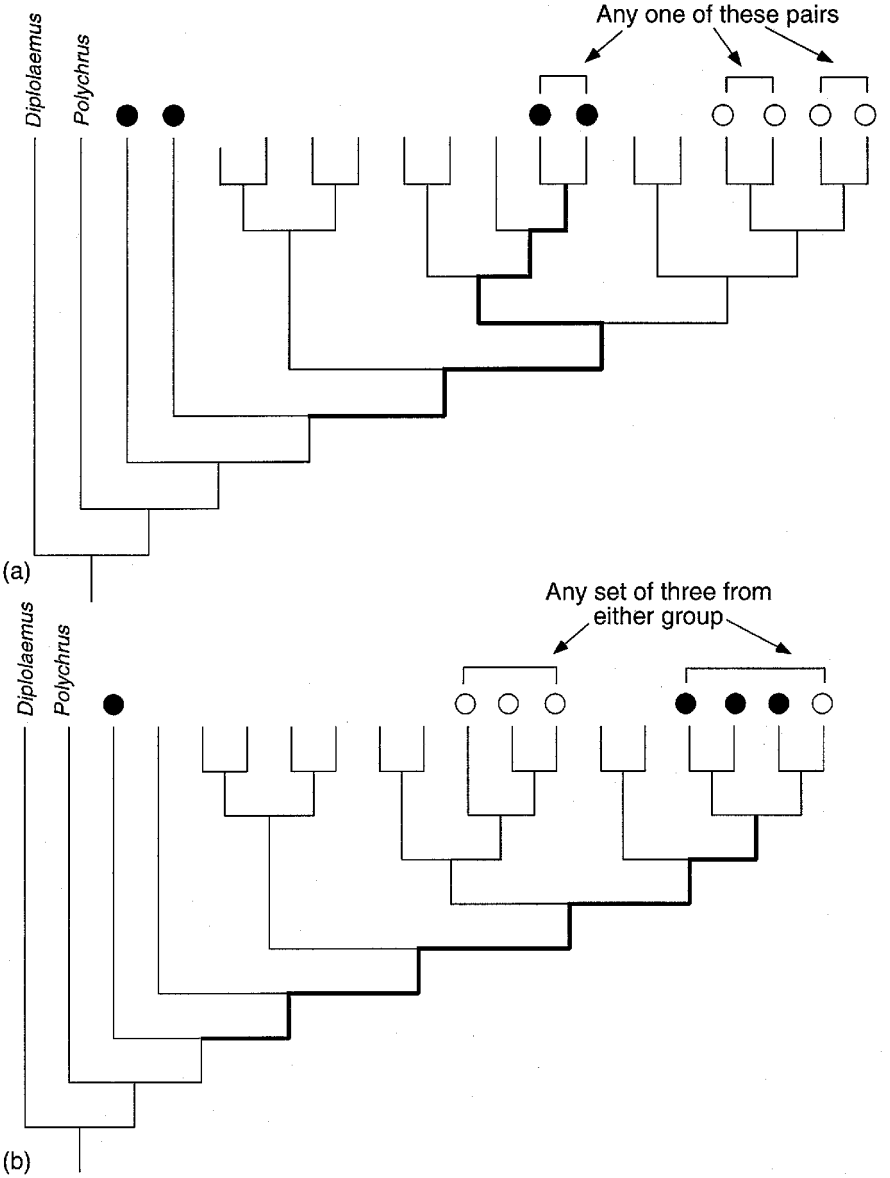

FIGURE 13. Criteria for subsampling species to generate a null distribution of bootstrap values and decay indices for permutation testing. (a) Four in-group species were chosen: a nested clade and its two most distant relatives within the in-group. (b) Four species were chosen: three representing a nested clade, and a fourth representing their most distantly related in-group clade. The nested clade is chosen to maximize the number of nodes crossed in connecting it to its most distant in-group relative. Filled circles denote species chosen for the subsample; the branches of interest are marked in bold. Open circles denote other possible choices.

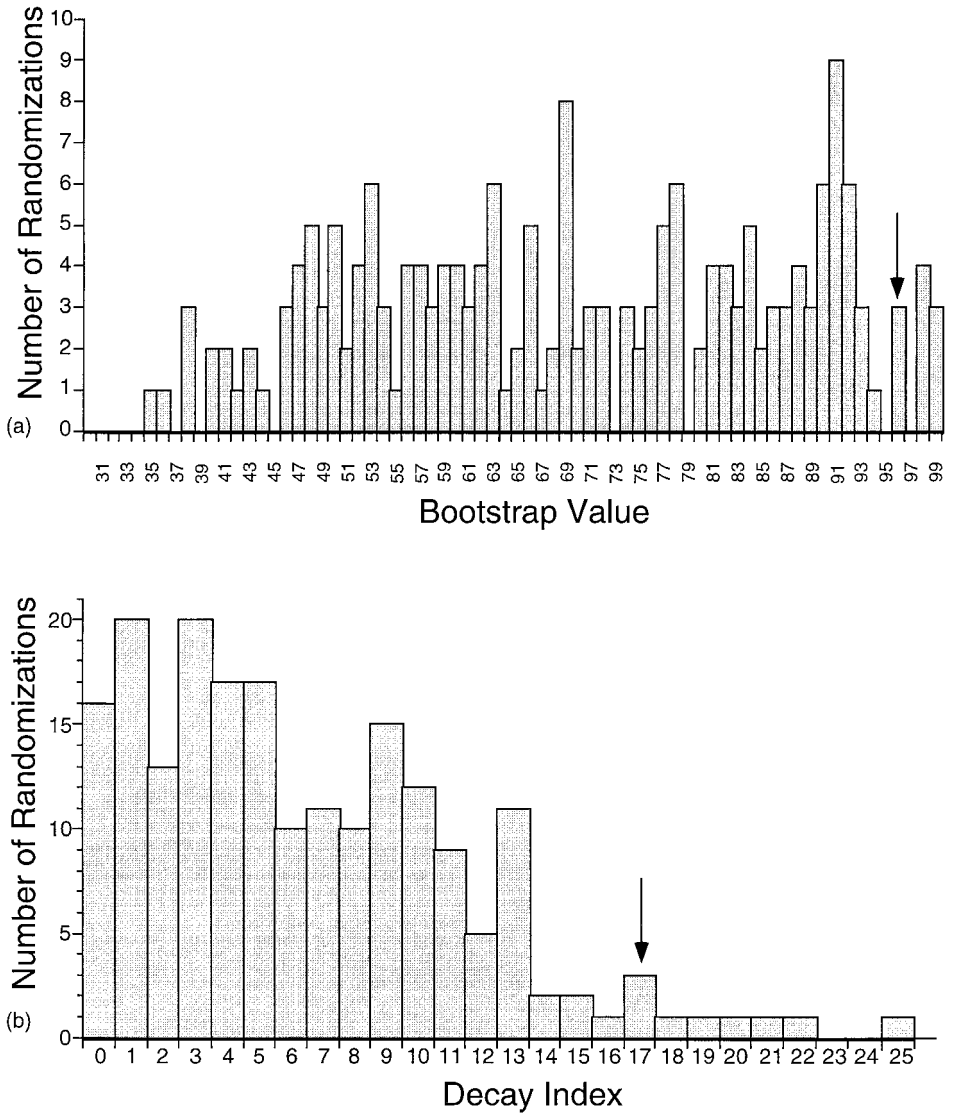

FIGURE 14. Distributions of bootstrap percentages (a) and decay indices (b) for focal branches of six-species trees generated by using phylogenetically randomized data. Silent transitions were excluded from the analysis, and 100 randomizations of the data were used. These values were obtained by using sampling of species as shown in Figure 13. Critical values corresponding to the 5% level of significance are indicated by arrows (96% and 17 for the bootstrap value and decay index, respectively).

TABLE 5. Tests for phylogenetic signal in branches connecting 17 major clades of anoles and using subsampling of taxa (see Figs. 11 and 15).

	A Permutation	B Parametric bootstrap	C Four-species	D Four-species
Data set	Silent transitions removed	All data	Silent transitions removed	All data
Null distribution	Best tree from randomized data	Exhaustive sampling of simulated data	Analytical; assumes molecular clock	Analytical; assumes molecular clock
Critical value of decay index	17	13	12–15[c]	12–15[c]
Number of significant subsamples (no. of total subsamples)	212 (2,380)	352 (2,380)	168 (680)	173 (680)
Conflicts with most-parsimonious tree[a]	4	33	42	71
Expected number of significant subsamples[b]	119	119	34	34

[a]Number of statistically significant subsamples in conflict with the most-parsimonious tree that includes all taxa (Fig. 7).
[b]Five percent of the total number of subsamples.
[c]Significance level varies according to numbers of informative characters (Table 1).

bootstrap considered all decay indices for each simulated data set. Different critical values and sampling of characters therefore were used in our statistical tests, based on the results of permutation versus the parametric bootstrap.

For both the permutation tests and parametric bootstrap tests, we observed a larger number of statistically significant subsamples from our data than were expected from the null distributions. For the permutation tests using unrooted trees, we observed 212 statistically significant subsamples, whereas 119 (5% of the number of possible subsamples) would have been expected from phylogenetically randomized data (Table 5). Likewise, for the parametric bootstrap analysis, we observed 352 statistically significant subsamples, whereas only 119 would have been expected from phylogenetically randomized data (Table 5).

To evaluate the statistical significance of the observed numbers of significant subsamples, we simulated 100 data sets for a hard polytomy with 17 in-group taxa and 2 out-groups, as had been used in our analyses of the anole data. The evolutionary model (branch lengths, base frequencies, and gamma and kappa parameters) used in the simulations was based on our anole data. Each simulated data set was subsampled according to the same scheme that had been applied to the anole data (Fig. 13) and yielded a total of 2,380 possible subsamples. The largest number of significant subsamples obtained for a simulated data set was 137, considerably less than the values obtained for the anole data. Therefore, we conclude that the values obtained for anoles are significant at $P < 0.01$.

To identify which combinations of branches contained the greatest amount of phylogenetic signal leading to rejection of the hypothesis of hard polytomy, we examined significant subsamples consistent with the most-parsimonious tree to determine which internal branches appeared more often than expected by chance. The observed number of times that each branch appeared in significant subsamples was compared with the number expected according to the frequency of occurrence of that branch in all subsamples. Branches appearing in significant subsamples more frequently than predicted from their proportional representation in all subsamples were highlighted as probable sources of phylogenetic signal. An exact statistical test for representation of particular branches in significant subsamples was not conducted because the observed numbers for different branches were not statistically independent.

Results of tests based on permutation and the parametric bootstrap led to qualitatively similar conclusions regarding locations of phylogenetic signal in the tree (Tables 5 and 6, Fig. 15). Both permutation and parametric bootstrap analyses indicated that branches 2 (grouping taxa 4–6), 6 (grouping taxa 7–17), 13 (grouping taxa 12–14), and 14 (grouping taxa 13–14) had greater than expected phylogenetic signal (Table 6). Branch 6 also received support from the addition of morphological characters to the phylogenetic analysis (Fig. 10). The permutation test highlighted branch 10 (grouping taxa 7–8), and the parametric bootstrap branch 12 (grouping taxa 9–10), as having greater than expected phylogenetic signal. These analyses were done on unrooted trees and therefore are not able to address the position of the root (branch 4 in Fig. 15 and Table 6). Combinations of branches marked with letters on Figure 15 appear to be disproportionate sources of phylogenetic signal.

Four-species tests of simultaneous branching.—Four-species tests were performed with *Polychrus acutirostris* as an out-group along with samples of three in-group species, both using all molecular data and also with silent transitions removed (Tables 5 and 7). These tests showed a higher proportion of conflicts with the most-parsimonious tree (Fig. 7) than did the tests based on permutation and the parametric bootstrap (Tables 5 and 6) because of ambiguity in rooting the tree; a separate "island" of trees (Maddison, 1991) exists that is one step longer than the most-parsimonious tree, with the root placed at branch 13 of Figure 15. Both analyses, using the criteria discussed above, reject the hypothesis of hard polytomy ($P < 0.01$).

TABLE 6. Distribution of branches in significant subsamples for permutation and parametric bootstrap tests. Branches denoted by a plus are represented more often than expected in significant subsamples

Branch no.	Percentage[a]	Permutation		Parametric bootstrap	
(Figure 15)		Expected[b]	Observed	Expected	Observed
1	2.1	11.34	0	19.36	0
2	5.4	29.16	+ 79	49.79	+ 90
3	2.1	11.34	10	19.36	8
4[c]	-	-	-	-	-
5	5.4	29.16	3	49.79	11
6	16.3	88.02	+134	150.29	+200
7	20.0	108.00	97	184.40	180
8	16.3	88.02	64	150.29	146
9	13.1	70.74	43	120.78	88
10	2.1	11.34	+ 21	19.36	7
11	5.4	29.16	13	49.79	36
12	2.1	11.34	9	19.36	+ 22
13	5.4	29.16	+ 38	49.79	+ 95
14	2.1	11.34	+ 29	19.36	+ 39
15	2.1	11.34	0	19.36	0
Totals	99.9	539.46	540	921.08	922

[a]The percentage of times each branch is present in all possible subsamples.
[b]The number of times each branch is expected to be included in significant subsamples, based on the percentages in column 2.
[c]Branch not included in unrooted trees.

To identify disproportionate sources of phylogenetic signal, we employed criteria analogous to those used in the permutation and parametric bootstrap analyses. In agreement with the permutation and parametric-bootstrap analyses, branches 2 and 14 received a larger number of significant results than was predicted based

TABLE 7. Distribution of branches in significant subsamples for four-taxon tests. Branches denoted by a plus are represented more often than expected in significant subsamples.

Branch no.	Percentage[a]	No. silent transitions		All data	
(Figure 15)		Expected[b]	Observed	Expected	Observed
1	1.0	2.89	2	2.64	0
2	2.8	8.09	+ 29	7.39	+ 15
3	1.0	2.89	+ 9	2.64	1
4	7.9	22.83	+ 49	20.86	+ 28
5	18.1	52.31	49	47.78	+ 65
6	21.8	63.00	57	57.55	56
7	19.1	55.20	36	50.42	35
8	10.9	31.50	13	28.78	8
9	7.9	22.83	12	20.86	8
10	1.0	2.89	+ 6	2.64	3
11	2.8	8.09	8	7.39	+ 22
12	1.0	2.89	+ 6	2.64	+ 13
13	2.8	8.09	+ 9	7.39	6
14	1.0	2.89	+ 4	2.64	+ 4
15	1.0	2.89	0	2.64	0
Totals	100.1	289.28	289	264.26	264

[a]The percentage of times each branch is present in all possible subsamples.
[b]The number of times each branch is expected to be included in significant subsamples, based on the percentages in column 2.

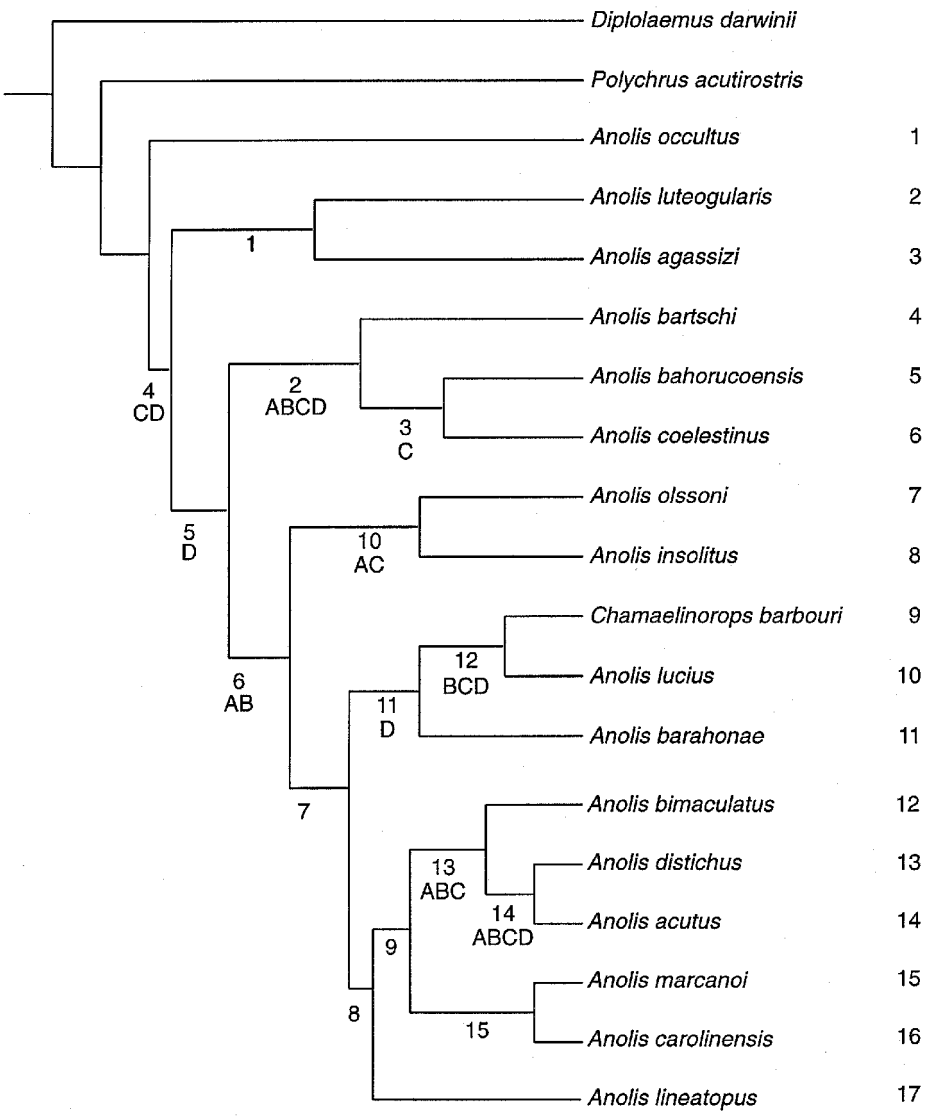

FIGURE 15. Results of tests for phylogenetic signal among branches connecting 17 major clades of anoles determined by using subsampling of species. Branches that appeared in significant subsamples more frequently than expected according to the criteria in Tables 6 and 7 are denoted as follows: A = permutation test, B = parametric bootstrap test, C = four-species test with silent transitions removed, D = four-species test including all data. Numbered branches correspond to those in Tables 6 and 7.

on the representation of these branches in the subsamples analyzed. Our results indicate the presence of disproportionate phylogenetic signal also for branches 4, 11, and 12 on Figure 15 (Table 7). The branch (branch 4 in Table 7) rooting the tree at *Anolis occultus* (taxon 1 in Fig. 15) contributes phylogenetic signal according to this analysis; placement of the root was not addressed by the permutation test and parametric bootstrap test, which used unrooted trees.

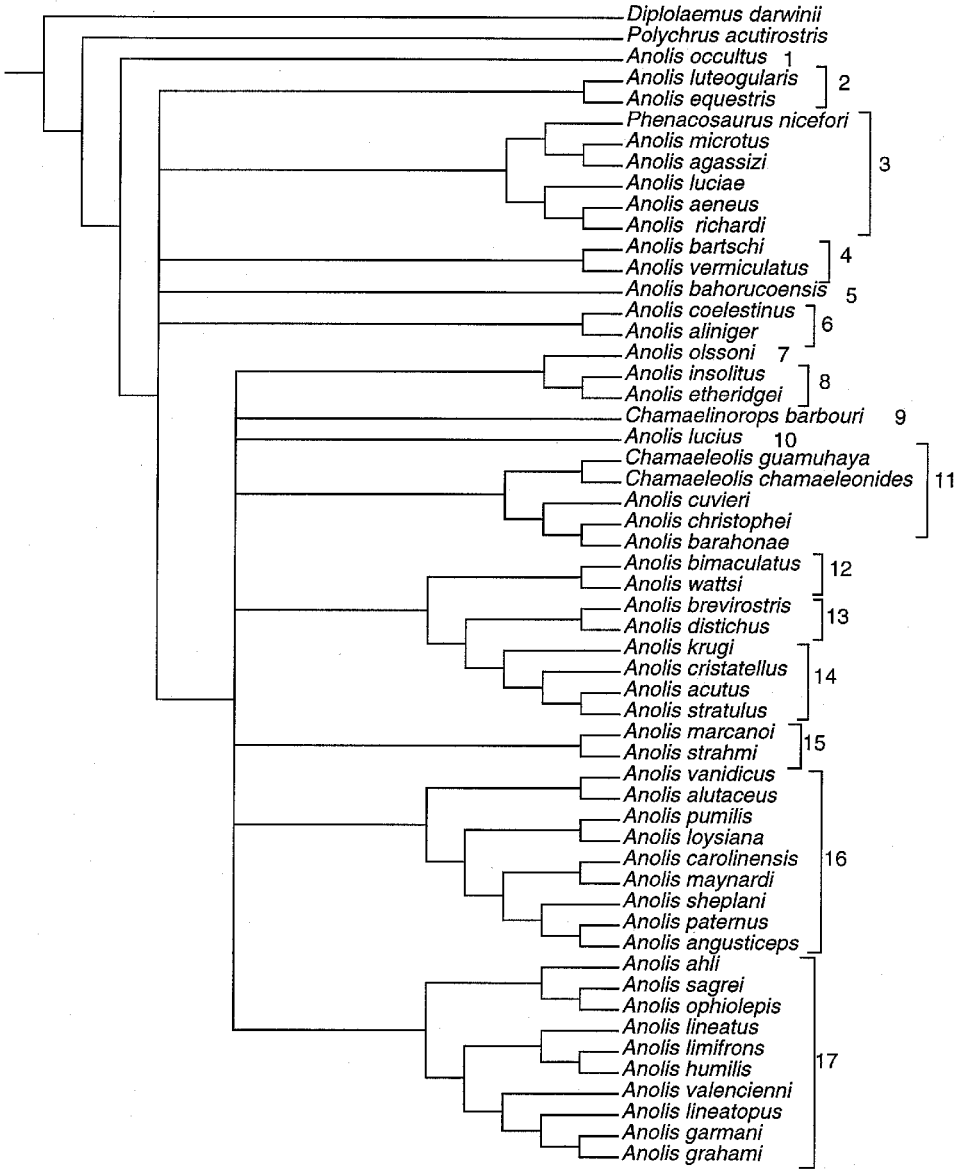

FIGURE 16. Test of skewness of tree lengths determined by using a constraint tree with 10 nodes collapsed from the most-parsimonious tree. Collapsed nodes have decay indices of 1 and bootstrap values < 30%. All nodes shown as dichotomous branches are constrained. Branches connected at polytomies are free to vary, and only these branches are involved in the g_1 test. The frequency distribution of total lengths of 10,000 randomly selected trees consistent with these constraints is not significantly skewed ($g_1 = -0.129$), as shown with a conservative test using 11 taxa and 500 variable characters (Hillis and Huelsenbeck, 1992).

Additional tests for phylogenetic structure.—A permutation test comparing the lengths of trees obtained from the randomized data set with that of the most-parsimonious tree obtained from an analysis of the actual data (the 17 taxa shown in Fig. 15) showed a significant phylogenetic structure, with all 100 randomizations giving longer trees

than the real data do. Results were significant whether silent transitions were included or removed. Similarly, with all 55 species and variable sites included, but constraining the clades as suggested by Hillis (1991) to allow only the 17 taxa representing the null hypothesis of a hard polytomy to vary (= 15 nodes collapsed from the most-parsimonious tree), the frequency distribution of tree lengths for 10,000 randomly sampled trees was significantly skewed ($g_1 = -0.256$, $P < 0.01$). Frequency distributions remained significantly skewed as the number of nodes collapsed from the most-parsimonious tree was decreased in single steps from 15 to 11 in order of decreasing bootstrap value but became insignificant when only 10 nodes remained collapsed ($g_1 = -0.129$) (Fig. 16).

Our use of the critical values from Hillis and Huelsenbeck (1992) is conservative because we used significance values estimated for 500 variable characters, whereas 523 variable characters exist for the 17 taxa free to vary. Critical values are proportional to the total number of possible trees, which has a simple relationship to the number of taxa in a tree (Table 2 of Hillis and Huelsenbeck, 1992) only when phylogenetic constraints are not used. Significance values for constrained trees were interpolated from Table 2 of Hillis and Huelsenbeck (1992) based upon the total number of possible trees (Table 13-2 of Hillis, 1991). For example, for our constrained tree with 10 nodes collapsed, the number of possible trees was 127,702,575, which is between the corresponding values of unconstrained trees having 11 (34,459,425 trees) and 12 taxa (654,729,075 trees).

Discussion

Phylogeny and Taxonomy of Anoles

Although many deep branches in the tree are not well supported, several previous phylogenetic hypotheses for anoles can be rejected statistically. In the most-parsimonious tree, *Anolis* as traditionally recognized is paraphyletic with respect to *Chamaelinorops*, *Chamaeleolis*, and *Phenacosaurus* (Fig. 7). This tree is significantly more parsimonious than the shortest tree that showed monophyly of the traditional genus *Anolis* (Table 4). Guyer and Savage's (1986, 1992) restricted genus *Anolis* also can be rejected as a monophyletic group. Monophyly of the other genera recognized by these authors cannot be rejected statistically, but only one of them, *Norops* (traditionally the beta section of *Anolis*), formed a monophyletic group in our most-parsimonious tree. Monophyly was rejected also for groups of anoles occurring on Cuba, Hispaniola, and Puerto Rico (Table 4). The most-parsimonious tree showed monophyly of Jamaican anoles, although the shortest tree showing nonmonophyly of this group cannot be rejected by our data.

Results of earlier studies appear not to contain any strong conflicts with our phylogenetic results presented above. An allozymic study using slowly evolving loci (Burnell and Hedges, 1990) produced few internally consistent results and was undermined by the assumption, contradicted by our results, that *Chamaelinorops* is an outgroup to *Anolis*. Although the 16S mitochondrial DNA data (Hass et al., 1993) provide almost no well–supported branches among 25 species of anoles, subsamples of 8 taxa analyzed by Hass et al. (1993) to address the placement of *Chamaeleolis* within *Anolis* gave very high neighbor-joining bootstrap values for branches phylogenetically congruent with the results of our analysis. Immunological data (Shochat and Dessauer, 1981; Hass et al., 1993) are consistent with our results in showing extremely short deep branches and *Chamaeleolis* nested within *Anolis*.

Hass et al. (1993) suggested synonymizing *Chamaelinorops* and *Chamaeleolis* with *Anolis*, based on immunological and DNA sequence evidence. Our data suggest that *Phenacosaurus*, *Chamaelinorops*, and *Chamaeleolis* are all nested within *Anolis* and therefore all should be synonymized with *Anolis*. If taxonomic recognition is restricted to groups that are clearly monophyletic, the only alternative under the traditional nomenclatural system to recognizing all anoles as *Anolis* is to divide the genus into at least 17 genera, an option we do not recommend. Alternatively, under the phylogenetic approach to nomenclature proposed

by de Queiroz and Gauthier (1992), all of these names are dissociated from the taxonomic category genus, and therefore they all can be used (Anolis for the inclusive clade, and Chamaelinorops, Chamaeleolis, and Phenacosaurus for three of its subclades).

Testing Hypotheses of a Hard Polytomy

Our DNA sequence data, as well as combined analyses of previous data sets, suggest that weak support for deep branches in the anole phylogenetic tree results from relatively rapid diversification early in the evolution of the group. However, our analyses provide evidence for sequential branching, in which relatively short times between sequential branching events sum to a substantially longer interval.

Phylogenetic analyses incorporating subsampling of species can identify cases in which including all species in the phylogenetic analysis produces the appearance of simultaneous branching of lineages when the branching is actually sequential. Subsampling of species permits rejection of the null hypothesis of a hard polytomy, although it is unable to provide statistical support for individual branches in the tree with all sampled taxa. This procedure is analogous to multiple-comparison tests such as the Tukey test used in an analysis of variance to examine multiple group means in pairwise comparisons (Zar, 1984:185). The null hypothesis in this case is that of simultaneous branching with significant branches from subsamples being analogous to significant pairwise comparisons. Just as groups whose means are very similar are more difficult to distinguish statistically than are those whose means are very different, relationships involving taxa separated by few characters or nodes are more difficult to distinguish statistically than are those involving taxa separated by many characters or nodes.

Tests based on subsampling of species indicate strong phylogenetic signal from the summation of branches identified in Tables 5 and 6 and Figure 15. These branches collectively represent a case in which sequential branching with short internodes produced, in the original parsimony analysis, the appearance of nearly simultaneous branching. In the four-species tests applied to the data with silent transitions removed, the root (branch 4 of Table 6 and Fig. 15) also contributed to rejection of the hypothesis of simultaneous branching. The four-species tests with silent transitions included were ambiguous regarding placement of the root, presumably because of noise contributed by saturated substitutions.

Because we have shown that substitutional saturation was not prevalent in our data when silent substitutions were eliminated, the skewness test for phylogenetic structure (Hillis, 1991; Hillis and Huelsenbeck, 1992) can serve to test the null hypothesis of a hard polytomy. By successively constraining branches in the order of decreasing bootstrap value (Hillis, 1991), we can reach a point at which the phylogeny becomes indistinguishable from a hard polytomy. The skewness test applied to our data revealed two polytomies, one involving five branches and the other involving eight branches (Fig. 16). These two groups are separated by the branch that groups taxa 7–17, which was identified in our other subsampling tests as contributing phylogenetic signal (branch 6 on Fig. 15 and Tables 5 and 6). However, the successive skewness tests are unable to identify among the remaining branches differences in phylogenetic signal revealed by our analysis of subsamples.

The ability to distinguish empirically between simultaneous branching of lineages and a sequential branching becomes more difficult as the internodes become shorter and the events studied move farther into the evolutionary past. If many lineages deep in the evolutionary history of anoles represent a rapid, sequential branching of species, then the phylogenetic disagreements observed among previous studies and the general inability of those studies to reveal strongly supported nodes at the base of the phylogenetic tree exemplify the general problem of recovering phylogenetic signal from short, relatively ancient branches. Two general conclusions result from this study: (1) We sometimes can find strong support for relationships that appear poorly supported in the context of the full tree; and (2) difficulties in finding strong support for

certain relationships can result from the age or the rapidity (or both) of successive splitting events. The methodology illustrated here for anoles should serve to explore the limits of phylogenetic resolution of comparative molecular data at various levels of evolutionary divergence.

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APPENDIX 1. MORPHOLOGICAL CHARACTERS, CHARACTERS STATES, AND STEP MATRICES USED FOR PARTIAL ORDERING OF CHARACTERS

1. Postfrontal bone (this study): 0 = present; 1 = absent.
2. Parietal foramen (Etheridge, 1959): 0 = at frontoparietal suture; 1 = entirely within parietal.
3. Parietal roof and crests (Etheridge, 1959): 0 = crests U-shaped (not meeting posteriorly), roof trapezoidal; 1 = crests V-shaped (meeting posteriorly at occipital crest but not forming midsagittal crest), roof triangular; 2 = crests Y-shaped (meeting posteriorly and forming midsagittal crest), roof triangular (ordered linear). These states refer to ontogenetic end points and are scored only from specimens close to maximum size.
4. Parietal posterior margin (Etheridge, 1959): 0 = notched posteriorly and not extending over occipital region as a bony half-funnel; 1 = not notched posteriorly with a half-funnel-shaped portion extending over occipital region (unordered). (Related to character 3 but treated separately because *Phenacosaurus* exhibits the derived condition of the parietal posterior margin while retaining the ancestral state of the parietal roof.) These states refer to ontogenetic end points and are scored only from specimens close to maximum size.
5. Dentary sculpturing (Etheridge, 1959): 0 = absent; 1 = present as irregular horizontal excavations (*cristatellus* type) on ventrolateral surface; 2 = present as a single, forward-directed, semilunar excavation (*cybotes* type) on ventrolateral surface; 3 = present as a series of 7–10 semilunar excavations (*krugi* type) on ventrolateral surface; 4 = present as irregular rugosities on posterolateral surface (*Chamaeleolis* type) (unordered).
6. Splenial bone (Etheridge, 1959): 0 = large and broad, includes anterior mylohyoid foramen and anterior inferior alveolar foramen but restricted to lingual face of mandible (e.g., *Polychrus*); 1 = small and narrow, extends from posterior border of single mylohyoid/alveolar foramen onto ventral surface of mandible (e.g., *A. griseus*); 2 = very small and narrow, restricted to lingual surface of mandible just posterior to single mylohyoid/alveolar foramen (e.g., *A. cuvieri*); 3 = very small and narrow, restricted to ventral surface of mandible (e.g., *A. bimaculatus*); 4 = absent (e.g., *A. sagrei*) (ordered by step matrix, Table A1).
7. Angular bone (Etheridge, 1959): 0 = present; 1 = absent.
8. Palatine teeth (Etheridge, 1959): 0 = present; 1 = absent.
9. Modal number of presacral vertebrae (Etheridge, 1959): 0 = 27; 1 = 26; 2 = 25; 3 = 24; 4 = 23; 5 = 22 (ordered linear).
10. Modal number of lumbar vertebrae (Etheridge, 1959): 0 = 0; 1 = 1; 2 = 2; 3 = 3; 4 = 4; 5 = 5; 6 = 6 (ordered linear).
11. Modal number of caudal vertebrae anterior to first autotomic vertebra (Etheridge, 1959): 0 = 11; 1 = 10; 2 = 9; 3 = 8; 4 = 7; 5 = 6; 6 = 5 (ordered: linear).
12. Caudal autotomy septa (Etheridge, 1959): 0 = present; 1 = absent.
13. Postxiphisternal inscriptional rib formula (attached chevrons:floating chevrons) (Etheridge, 1959, 1965): 0 = 4:3; 1 = 5:1; 2 = 4:2; 3 = 5:0; 4 = 4:1; 5 = 3:2; 6 = 4:0; 7 = 3:1; 8 = 2:2; 9 = 1:3 (ordered by step matrix, Table A2). Modal condition is given.
14. Caudal ribs/transverse processes (Etheridge, 1959, 1967): 0 = present on caudal vertebrae extending into posterior half of series with posterior-most processes oriented laterally, autotomy septa (when present) posterior to processes ("*Sceloporus* type");

1 = disappear after a short series confined to caudal vertebrae on anterior half of tail with the posterior-most processes oriented laterally, autotomy septa (when present) anterior to processes ("alpha type"); 2 = present on caudal vertebrae extending into posterior half of series with a posterior series of processes oriented anterolaterally, autotomy septa (when present) anterior to processes ("beta type"); 3 = extend into posterior half of caudal series with the processes oriented laterally and expanded distally ("*Chamaelinorops* type") (unordered).

15. Interclavicle (Etheridge, 1959): 0 = lateral processes in contact with clavicles for less than half their lengths ("arrow-shaped"); 1 = lateral processes in contact with clavicles for more than half their lengths ("T-shaped").
16. Parabasisphenoid (this study): 0 = basipterygoid processes lack anteroventral crests; 1 = basipterygoid processes with anteroventral crests that approach or meet one another medially, forming a shelf below the parasphenoid rostrum.

TABLE A1. Step matrix for splenial.

	0	1	2	3	4
0	0	1	2	2	3
1	1	0	1	1	2
2	2	1	0	1	1
3	2	1	1	0	1
4	3	2	1	1	0

TABLE A2. Step matrix for postxiphisternal inscriptional ribs.

	0	1	2	3	4	5	6	7	8	9
0	0	2	1	3	2	2	3	3	3	3
1	2	0	1	1	1	2	2	2	3	4
2	1	1	0	2	1	1	2	2	2	3
3	3	1	2	0	1	2	1	2	3	4
4	2	1	1	1	0	1	1	1	2	3
5	2	2	1	2	1	0	2	1	1	2
6	3	2	2	1	1	2	0	1	2	3
7	3	2	2	2	1	1	1	0	1	2
8	3	3	2	3	2	1	2	1	0	1
9	3	4	3	4	3	2	3	2	1	0

(Appendix 2 is shown on the next page.)

APPENDIX 2. MORPHOLOGICAL CHARACTER STATE MATRIX

Character numbers correspond to the characters as described in Appendix 1. Characters 3, 9, 10, and 11 are ordered characters. The splenial bone (6) and the inscriptional rib formula (13) are partially ordered with the use of step matrices to reflect the possible transitions from one state to another (Appendix 1). All other characters are treated as unordered. Morphological data are not available for *Chamaeleolis guamuhaya.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Diplolaemus darwinii	0	0	0	0	0	0	0	1	3	0	2	0	5	0	1	?
Polychrus acutirostris	1	0	0	0	0	0	0	1	0	1	?	1	?	1	0	0
Anolis acutus	0	0	2	1	0	2	1	1	3	4	5	0	7	1	0	0
Anolis cristatellus	0	1	2	1	1	(12)	1	1	4	4	5	0	8	(12)	0	1
Anolis krugi	0	1	2	1	3	1	1	1	4	(34)	5	0	8	1	0	1
Anolis stratulus	0	0	2	1	0	(34)	1	1	3	4	5	0	7	1	0	0
Anolis bimaculatus	0	0	2	1	0	3	1	1	3	4	5	0	7	1	0	0
Anolis wattsi	0	0	2	1	0	(34)	1	1	3	4	5	0	7	1	1	0
Anolis bahorucoensis	0	1	2	1	0	1	1	1	3	4	4	0	7	1	1	0
Anolis insolitus	1	1	(12)	1	0	4	1	?	3	(45)	?	1	7	1	1	0
Anolis etheridgei	0	1	2	1	?	4	1	1	3	6	(34)	0	7	1	1	0
Anolis lineatus	0	0	2	1	0	4	1	1	3	3	4	0	8	2	1	0
Anolis garmani	0	1	2	1	0	4	1	1	3	3	4	0	7	2	1	0
Anolis lineatopus	0	1	2	1	0	4	1	1	3	3	4	0	7	2	1	0
Anolis grahami	0	1	2	1	0	4	1	1	3	3	(45)	0	7	2	1	0
Anolis valencienni	0	0	2	1	0	4	1	1	3	3	4	0	8	2	1	0
Anolis sagrei	1	0	2	1	0	4	1	1	3	4	6	0	8	2	1	1
Anolis ahli	0	0	2	1	0	4	1	1	3	(34)	(56)	0	8	2	1	1
Anolis ophiolepis	1	0	2	1	0	4	1	1	3	(34)	5	0	8	2	1	0
Anolis limifrons	0	0	1	1	0	4	1	1	3	4	4	0	7	2	1	0
Anolis humilis	0	0	1	1	0	4	1	1	4	(34)	5	0	8	2	1	0
Anolis olssoni	1	0	2	1	0	4	1	1	3	5	4	0	7	1	1	0
Chamaelinorops barbouri	1	0	0	?	0	4	1	1	3	5	?	1	4	3	1	0
Anolis microtus	?	0	0	0	0	1	1	1	3	3	(0123)	0	3	1	0	?
Phenacosaurus nicefori	0	0	0	?	0	1	1	1	4	(23)	?	1	1	1	0	0
Anolis richardi	0	0	0	0	0	1	1	1	3	(45)	3	0	6	1	0	0
Anolis aeneus	0	0	0	0	0	1	1	1	3	4	3	0	6	1	0	0
Anolis luciae	0	0	0	0	0	1	1	1	3	4	3	0	6	1	0	0
Anolis agassizi	0	0	0	0	0	1	1	1	3	4	3	0	6	1	0	0
Anolis brevirostris	0	0	2	1	0	(12)	1	1	3	4	5	0	7	1	0	0
Anolis distichus	0	0	2	1	0	1	1	1	3	3	5	0	7	1	0	0
Anolis marcanoi	1	0	2	1	2	4	1	1	3	(34)	5	0	8	1	0	0
Anolis strahmi	1	0	2	1	2	4	1	1	3	3	5	0	8	1	0	0
Anolis christophei	0	0	(12)	1	0	4	1	1	3	4	5	0	7	1	1	0
Anolis coelestinus	0	0	?	0	0	4	1	1	3	4	4	0	6	1	1	0
Anolis aliniger	0	0	(12)	0	0	4	1	1	3	4	4	0	7	1	1	0
Anolis bartschi	0	0	2	1	0	4	1	1	3	4	5	0	7	1	1	0
Anolis vermiculatus	0	1	2	1	0	4	1	1	3	3	(45)	0	7	1	1	0
Anolis lucius	0	0	2	1	0	4	1	1	3	4	5	0	7	1	1	0
Anolis pumilis	1	0	1	1	0	4	1	1	3	5	5	0	8	1	1	0
Anolis loysiana	0	0	2	1	0	4	1	1	3	(45)	4	0	8	1	1	0
Anolis paternus	0	0	2	1	0	4	1	1	3	5	4	0	8	1	1	0
Anolis sheplani	0	1	(12)	1	0	4	1	1	3	4	?	1	1	1	1	0
Anolis angusticeps	0	0	2	1	0	4	1	1	3	(45)	4	0	8	1	1	0
Anolis carolinensis	0	0	2	1	0	4	1	1	3	4	4	0	7	1	1	0
Anolis maynardi	0	0	2	1	0	4	1	1	3	4	4	0	7	1	1	0
Anolis vanidicus	0	1	0	1	0	4	1	1	3	5	4	?	7	1	1	0
Anolis alutaceus	0	0	2	1	0	4	1	1	3	(56)	4	0	7	1	1	0
Anolis barahonae	0	0	2	1	0	2	1	1	3	3	3	0	7	1	0	0
Anolis cuvieri	0	0	2	1	0	2	1	1	3	3	3	0	7	1	0	0
Chamaeleolis chamaeleonides	0	0	2	1	4	1	0	0	3	3	?	1	2	1	0	0
Anolis occultus	0	0	0	0	0	4	1	1	4	(34)	2	0	3	1	1	0
Anolis luteogularis	0	0	2	1	0	1	1	1	3	3	(12)	0	7	1	1	0
Anolis equestris	0	0	2	1	0	1	1	1	3	3	(12)	0	7	1	1	0