JOURNAL OF EXPERIMENTAL ZOOLOGY (MOL DEV EVOL) 294:1–16 (2002)

Molecular Phylogenetic Perspective on Evolution of Lizards of the *Anolis grahami* Series

TODD R. JACKMAN,[1]* DUNCAN J. IRSCHICK,[2] KEVIN DE QUEIROZ,[3] JONATHAN B. LOSOS,[4] AND ALLAN LARSON[4]
[1]*Department of Biology, Villanova University, Villanova, Pennsylvania 19085*
[2]*Department of Ecology and Evolutionary Biology, Tulane University, New Orleans, Louisiana 70118–5698*
[3]*Division of Amphibians and Reptiles, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560–0162*
[4]*Department of Biology, Washington University, St. Louis, Missouri 63130–4899*

ABSTRACT We report the results of phylogenetic analyses of 1447 bases of mitochondrial DNA sequence for 21 populations representing seven species of the *Anolis grahami* series (*A. conspersus*, *A. garmani*, *A. grahami*, *A. lineatopus*, *A. opalinus*, *A. reconditus*, and *A. valencienni*), six of which occur on Jamaica. These data include 705 characters that are phylogenetically informative according to parsimony. A parsimony analysis of these data combined with previously published allozymic data yields a single most parsimonious tree with strong support for monophyly of the *A. grahami* series, the sister-group relationship between *Anolis lineatopus* and *A. reconditus* and a clade composed of *Anolis garmani*, *A. grahami*, and *A. opalinus*. Based on DNA data alone, *A. conspersus* is nested within *A. grahami*. Haplotypes sampled from geographic populations of *A. grahami, A. lineatopus,* and *A. opalinus* are highly divergent (≈12–15% sequence difference on average for each species) and show similar phylogeographic patterns, suggesting that each of these currently recognized species may be a complex of species. *Anolis valencienni* also shows high sequence divergence among haplotypes from different geographic populations (≈8% sequence difference) and may contain cryptic species. Divergence among haplotypes within *A. garmani* is substantially lower (≈3% sequence difference), and phylogeographic patterns are significantly different from those observed in *A. grahami, A. lineatopus* and *A. opalinus. J. Exp. Zool. (Mol. Dev. Evol.) 294:1–16, 2002.*

For more than 30 years, *Anolis* lizards of the West Indies have been a focus of research regarding habitat use (Rand, '64; Schoener, '68; Losos, '90a), ecomorphology (Rand and Williams, '69; Moermond, '79; Losos, '90a), functional and behavioral characteristics (Moermond, '81; Losos, '90b; Irschick and Losos, '98), and systematics (reviewed by Jackman et al., '97, '99). A phenomenon that has received considerable attention is the evolution of morphologically distinct habitat specialists, termed "ecomorphs," on each of the Greater Antillean islands (Cuba, Hispaniola, Puerto Rico, and Jamaica), with each island exhibiting similar sets of ecomorphs (Williams, '83; Losos, '92; Losos et al., '98).

Losos ('92) reconstructed ecomorphological evolution for Puerto Rican and Jamaican (*A. grahami* series) anoles using phylogenetic information from Hedges and Burnell ('90) for the *Anolis grahami* series (Fig. 1). Because phylogenetic relationships are not entirely well resolved by the earlier study, new data and phylogenetic analyses are needed to clarify relationships among Jamaican anoles. Here, we examine phylogenetic relationships within the *Anolis grahami* series, which includes *A. conspersus* from Grand Cayman plus all Jamaican anoles except *A. sagrei* (Etheridge, '59; Williams, '76; Savage and Guyer, '89). *Anolis lineatopus* contains four named geographic races that differ in scalation, dewlap and body color, and body size (Underwood and Williams, '59). Relationships among these races are examined, and the result-

Grant Sponsor: National Science Foundation (NSF DEB 9318642); Grant sponsor: David and Lucile Packard Foundation.
*Correspondence to: Dr. Todd R. Jackman, Department of Biology, Villanova University, Villanova, PA 19085.
E-mail: todd.jackman@villanova.edu
Received 28 June 2001; Accepted 11 December 2001
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/jez.10073

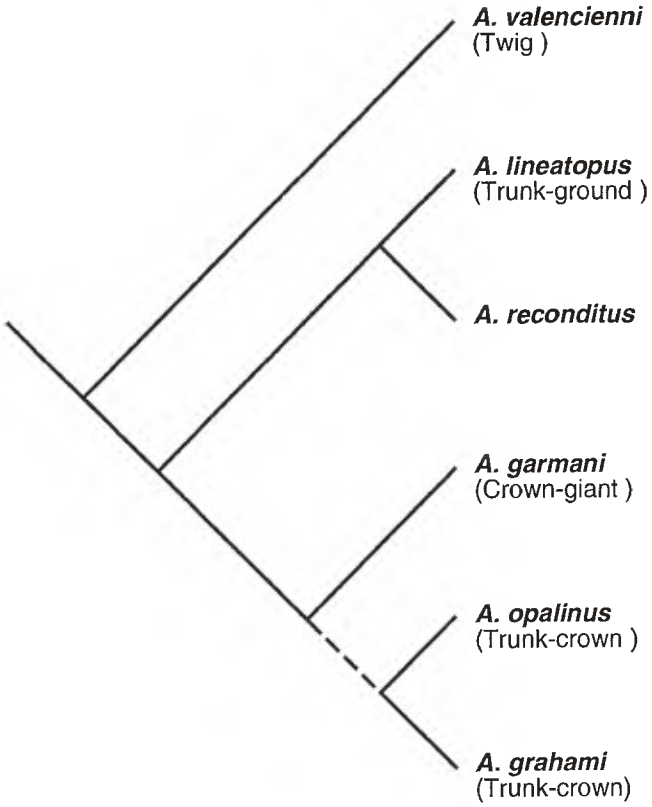

Fig. 1. Phylogenetic hypothesis reported for Jamaican anoles in an allozymic study by Hedges and Burnell ('90). Hedges and Burnell ('90) proposed an unresolved trichotomy between *A. garmani*, *A. grahami* and *A. opalinus*. Losos ('92) resolved the trichotomy as shown (branch represented by the dashed line) using derived morphological similarities shared by *A. grahami* and *A. opalinus* (Hedges and Burnell, '90). Ecomorph categories are shown for species that have been assigned one of the standard ecomorphs (Jackman et al., '97; Losos et al., '98).

ing area cladogram is tested for congruence with those obtained for several geographically co-distributed species on Jamaica (*A. garmani*, *A. grahami*, *A. opalinus*).

Mitochondrial DNA sequences provide many informative characters for phylogenetic analysis and thus constitute useful data for examining relationships among Jamaican anole species. In addition to gathering mitochondrial DNA sequences, we reanalyze allozymic data of Hedges and Burnell ('90), both separately and combined with our DNA sequence data, and compare these two data sets for their ability to discriminate alternative phylogenetic hypotheses.

MATERIALS AND METHODS

Specimens examined. We examined 24 specimens representing all currently recognized species and most subspecies of the *A. grahami* series plus fifteen anole outgroups. One individual was sampled for each of the four subspecies of *A. lineatopus* (Fig. 2). Sampling also included five individuals representing both subspecies of *A. grahami* (from Discovery Bay [2], Negril, Kingston, and Port Antonio), five individuals of *A. opalinus* (from Hardwar Gap, Quick Step, Kingston, Port Antonio, and Negril), four individuals of *A. garmani* (from Port Antonio, Negril, Discovery Bay, and Kingston), three individuals of *A. valencienni*, two individuals of *A. reconditus*, and one individual of *A. conspersus*.

The *A. grahami* series is part of the beta section of *Anolis* (Etheridge, '59; Williams, '76; *Norops* of Guyer and Savage, '86, '92). DNA data have corroborated monophyly of the beta section (Jackman et al., '99), originally diagnosed by anterolaterally oriented transverse processes of caudal vertebrae originating posterior to the autotomy septa (Etheridge, '59). Relationships among beta anoles are uncertain. Therefore, we included 13 additional beta anoles representing all of the other series of beta anoles recognized by Etheridge (*A. ahli*, *A. allogus*, *A. biporcatus*, *A. carpenteri*, *A. fuscoauratus*, *A. imias*, *A. lemurinus*, *A. lineatus*, *A. nitens*, *A. ophiolepis*, *A. ortoni*, *A. sagrei*, and *A. trachyderma*) and two Caribbean alpha anoles (*A. carolinensis* and *A. cristatellus*) as outgroups. Localities, catalogue numbers, and GenBank accession numbers for specimens whose DNA sequences are reported here for the first time are as follows: *A. allogus* Cuba (USNM 497959, AF294313), *A. biporcatus* Costa Rica (CAS 178135, AF294286), *A. carolinensis* Louisiana (JBL 982, AF294279), *A. carpenteri* (LSU

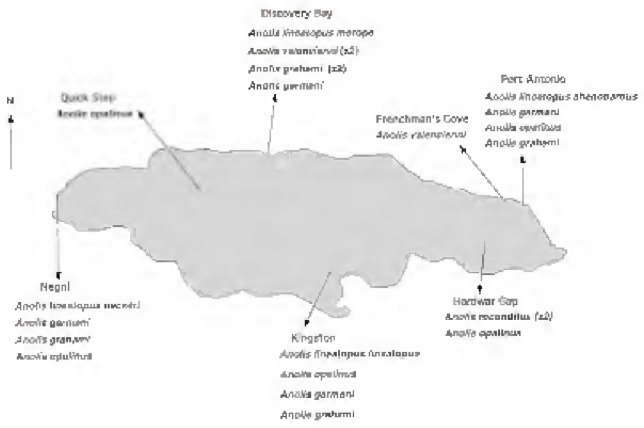

Fig. 2. Map showing geographic sampling of the *A. grahami* series on Jamaica. Multiple samples from the same locality are indicated by 'x2.' Samples of *Anolis grahami* represent two subspecies, *A. g. grahami* (Kingston, Negril, and Discovery Bay), and *A. g. aquarum* (Port Antonio).

H14688, AF294282), *A. conspersus* Grand Cayman (JBL 414, AF294304), *A. cristatellus* Puerto Rico (RT13038, AF294280), *A. fuscoauratus* Brazil (LSU 13566, AF294284), *A. garmani* Kingston (USNM 337569, AF294291), *A. garmani* Port Antonio (USNM 337568, AF294292), *A. garmani* Discovery Bay (USNM 337591, AF294289), *A. grahami* Negril (USNM 337583, AF294301), *A. grahami* Discovery Bay 1 (USNM 337582, AF294299), *A. grahami* Kingston (USNM 337577, AF294302), *A. imias* (USNM 191260, AF294314), *A. lemurinus* Costa Rica (USNM 326140, AF294283), *A. lineatopus ahenobarbus* Port Antonio (USNM 337596, AF294297), *A. lineatopus neckeri* Negril (USNM 337589, AF294296), *A. lineatopus lineatopus* Kingston (USNM 337609, AF294298), *A. nitens* (LSU H12298, AF294281), *A. opalinus* Port Antonio (USNM 337604, AF294306), *A. opalinus* Hardwar Gap (USNM 337603, AF294307), *A opalinus* Kingston (USNM 337614, AF294305), *A. opalinus* Quick Step (GM 27874, AF294308), *A. ortoni* (LSU H13904, AF294288), *A. reconditus* 1 Hardwar Gap (JBL 1, AF294293), *A. reconditus* 2 Hardwar Gap (USNM 337599, AF294294), *A. trachyderma* Brazil (LSU 14285, AF294285), *A. valencienni* Frenchman's Cove (USNM 337617, AF294312), *A. valencienni* Discovery Bay 1 (USNM 337617, AF294310). Data were obtained from Jackman et al. ('99) for *A. ahli* (USNM 497946, AF055941), *A. garmani* Negril (JBL 22-26, AF055937), *A. grahami* Discovery Bay 2 (JBL 250, AF055939), *A. lineatopus* Discovery Bay (JBL 248, AF055938), *A. lineatus* (LSU H5450, AF055936), *A. ophiolepis* (USNM 498059, AF055942), *A. sagrei* (JBL 407, AF055940), and *A. valencienni* Discovery Bay 2 (JBL 262, AF055953). Codes refer to collections of Jonathan Losos (JBL), Greg Mayer (GM), Richard Thomas (RT), United States National Museum of Natural History, Washington, DC (USNM), California Academy of Science, San Francisco (CAS), and Louisiana State University, Baton Rouge (LSU).

DNA laboratory protocols

Tissue samples were collected at field sites and stored in 100% ethanol at –80°C. Genomic DNA was extracted from muscle or liver using Qiagen QIAamp tissue kits. Amplification of genomic DNA was conducted using a denaturation at 94°C for 35 sec, annealing at 45°C –53°C for 35 sec, and extension at 70°C for 150 sec with 4 sec added to the extension per cycle, for 30 cycles. Amplified products were purified on 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., '82). Template DNA was eluted from acrylamide passively over three days in which Maniatis elution buffer (Maniatis et al., '82) was replaced each day. Cycle-sequencing reactions were run using a Promega fmol DNA sequencing system with a denaturation at 95°C for 35 sec, annealing at 53°C–61°C for 35 sec, and extension at 70°C for 1 min for 30 cycles. Sequencing reactions were run on Long Ranger sequencing gels for 4–12 hr at 38°C –42°C. Amplifications used primers depicted in Figure 3. Sequences of these primers are from Macey et al. ('97a,b) except for primer H4803, which is from Jackman et al. ('99). All primers were used for amplification and sequencing. We used negative controls during amplification.

DNA sequence alignment and character homology

We gathered DNA sequence data for a portion of the mitochondrial genome including genes encoding ND2, five transfer RNAs, and part of COI (Fig. 3). Sequences were aligned manually. We used MacClade (Maddison and Maddison, '92) to translate sequences encoding ND2 and COI into amino acids to check scoring and alignments. Sequences encoding tRNAs were aligned manually based on secondary structural models (Kumazawa and Nishida, '93; Macey and Verma, '97). Phylogenetic analyses were conducted using 1447 alignable positions. Because some parts of the sequence were difficult to align, we excluded sites 1055–1067, 1288–1307, and 1328–1335 from phylogenetic analyses.

Phylogenetic analysis of DNA and allozymic data

We performed three sets of phylogenetic analyses. First, we analyzed DNA data alone using parsimony and maximum-likelihood criteria. Second, we analyzed allozymic data alone using parsi-

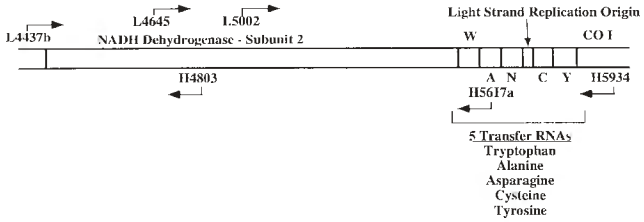

Fig. 3. Mitochondrial DNA region sequenced in this study. Primers are marked by their position in the human genome (Anderson et al., '81) and whether their extension produces the heavy (H) or light (L) strands of mitochondrial DNA.

mony. Finally, we performed a combined analysis of DNA and allozymic data using parsimony following Larson ('98). Many populations sampled for DNA sequence were not sampled for allozymes. Computer simulations by Wiens ('98) suggest that adding characters with incomplete taxon sampling usually increases phylogenetic accuracy but sometimes is misleading. We therefore conducted two different analyses of combined data, one in which we restricted our sampling to those samples for which both allozymic and DNA data were available and a second analysis that included all samples but with missing information for taxa lacking allozymic characters. Samples having complete data were *A. carolinensis*, *A. cristatellus*, *A. garmani* Discovery Bay, *A. grahami* Discovery Bay 1, *A. lineatopus* Discovery Bay, *A. opalinus* Quick Step, *A. reconditus* 1, *A. sagrei*, *A. valencienni* Discovery Bay 1, and *A. valencienni* Frenchman's Cove. Samples of *A. grahami* and *A. lineatopus* represent the same subspecies examined by Hedges and Burnell ('90). Searches for optimal trees from mitochondrial DNA data were conducted with PAUP*4.0b2 (Swofford, 2000) using random-addition heuristic searches with 100 replicates under parsimony and 20 replicates under likelihood. Tree bisection and reconnection (TBR) branch swapping was used for both parsimony and likelihood analyses. For the maximum-likelihood analysis, transition/transversion ratio and the gamma shape parameter (four categories of change) were estimated using the most parsimonious tree.

Allozymic data from Hedges and Burnell ('90) were analyzed using the frequency-parsimony method (Swofford and Berlocher, '87) described by Berlocher and Swofford ('97). This method uses a stepmatrix of Manhattan distances, which we generated using SYSTAT version 5.2.1, to incorporate allelic frequencies (as opposed to using only presence/absence of alleles). Recent studies have shown that frequencies contain substantial phylogenetic information (Wiens, '95). The frequency-parsimony method also treats allozymic loci as characters, thus enabling combination of allozymic and DNA data in a single parsimony analysis. For the allozymic analysis, we made the following modifications to the data of Hedges and Burnell ('90): 1) *Anolis cybotes* and *A. darlingtoni* and all alleles that were present only in these taxa were eliminated; 2) the frequency of *Mpi-1* allele e2 in *Anolis lineatopus* was corrected from 0.80 to 0.90 (SB Hedges, personal communication); 3) the allele for *Mpi-1* in *Anolis reconditus* was corrected from f1 (1.00) to e1 (1.00) (SB Hedges, personal communication); and 4) the frequencies of *Tpep* alleles e2 and f1 in *Anolis opalinus* were added as follows: e2 = 0.12, f1 = 0.88 (SB Hedges, personal communication). In addition, we constrained *A. carolinensis*, *A. cristatellus*, and *A. sagrei* to be outgroups. Allozymic data were subjected to a branch-and-bound search (using simple addition) to find the most parsimonious tree.

For all data sets, we calculated bootstrap values (Felsenstein, '85b; 1,000 replicates using random-addition heuristic searches with 10 replicates and TBR branch swapping) to assess support for each node. For trees based on DNA and combined data, we calculated decay indices ("branch support" of Bremer, '94) for each node using reverse constraints.

Hypothesis tests

We tested several phylogenetic hypotheses using mitochondrial DNA data and two different statistical methods: Wilcoxon signed-ranks tests (Templeton, '83) and parametric bootstrap tests (Huelsenbeck et al., '96). Wilcoxon signed-ranks tests, implemented using PAUP*4.0b.2 (Swofford, 2000), were conducted following Templeton ('83) but using the more conservative two-tailed probability values (Felsenstein, '85a). First, we tested whether the mitochondrial DNA tree was significantly more parsimonious for the DNA data, than either the hypothesis shown in Figure 1 or the tree generated from our own parsimony analysis of allozymic data. To compare the DNA tree to phylogenetic hypotheses derived from data sets with fewer taxa we used the backbone-constraint option in PAUP*; taxa present in both data sets are constrained to the topology specified by the allozymic or combined analyses as appropriate, whereas other taxa are free to vary when searching for the shortest tree. The most parsimonious trees compatible with alternative hypotheses were generated using a heuristic search in PAUP* with 50 random additions and constraints shown in Appendix 1. Wilcoxon signed-ranks tests were used also with allozymic and combined data sets. For alternative hypotheses represented by more than one equally most parsimonious topology, the most conservative result (highest P value) was reported.

For the parametric bootstrap test (Huelsenbeck et al., '96) of alternative phylogenetic hypotheses, the shortest tree compatible with each alternative hypothesis was obtained as described above for Wilcoxon signed-ranks tests. Using maximum-

likelihood estimation in PAUP*4.0b.2 (Swofford, 2000), the following parameters were estimated for the data on each alternative hypothesis: Six different rate parameters for every possible substitution type (general time-reversible model) and gamma distribution of the density of substitutions. Using these parameters and observed base composition of DNA sequences, maximum-likelihood estimates of branch lengths were obtained using PAUP* for trees representing each alternative hypothesis. Using these trees and branch lengths (Appendix 2), and parameters estimated above (base composition, transition/transversion ratio, gamma distribution shape, and kappa), 100 data sets were simulated for each alternative hypothesis using Seq-Gen program V1.1 (Rambaut and Grassly, '97). These data sets were equal in size to the actual data being analyzed (1,462 bases). Each simulated data set was analyzed to find both the most parsimonious tree for that data set and the most parsimonious tree compatible with the hypothesis used to generate the data set. Differences in length between the most parsimonious tree and the most parsimonious constrained tree obtained for each of the 100 data sets were used as a null distribution. The difference corresponding to the upper five percent of values was used as a critical value. The difference in length between the most parsimonious tree for the actual data being analyzed and the shortest tree corresponding to the alternative hypothesis being examined was compared to the critical value generated from simulations. The most parsimonious tree was considered significantly shorter than an alternative hypothesis if the difference in length between these trees exceeded the critical value. This parametric bootstrap test has been termed the SOWH test by Goldman et al. (2000).

RESULTS

Mitochondrial DNA data

The mitochondrial DNA sequences contain 1,447 alignable base positions of which 888 are variable and 705 are parsimony-informative. Support for nodes within the *A. grahami* series ranges from weak to strong, with 18 of 22 nodes having bootstrap values over 70% and decay indices over four (Fig. 4). The most parsimonious tree (4,223 steps; Fig. 4) divides the *A. grahami* series into two primary clades: One consists of *A. lineatopus* and *A. reconditus*, and the other one comprises the remaining members of the series (Figs. 4 and 5). *Anolis conspersus* is the sister taxon of *A. grahami*

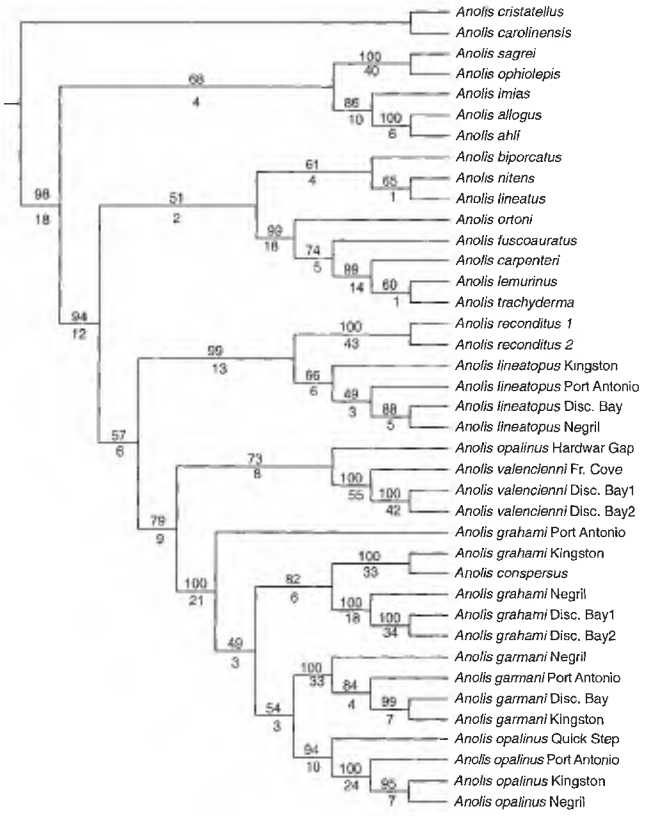

Fig. 4. The most parsimonious tree generated from the mitochondrial DNA sequences for fifteen outgroup anoles and 24 anoles of the *A. grahami* series (4,223 steps, 888 variable and 705 parsimony-informative characters). Numbers above branches are bootstrap values (1,000 replicates); numbers below branches are decay indices. Maximum-likelihood analysis of the same data produces an identical topology.

from Kingston, making *A. grahami* paraphyletic. *Anolis opalinus* from Hardwar Gap is the sister lineage of *A. valencienni* and not closely related to other populations of *A. opalinus*. In all other species represented by multiple populations (*A. valencienni*, *A. lineatopus*, and *A. garmani*), haplotypes from conspecific individuals form monophyletic groups. *Anolis valencienni* and Hardwar Gap *A. opalinus* together form the sister group of a clade composed of *A. grahami*, *A. garmani*, and the remaining *A. opalinus*. *Anolis garmani* and *A. opalinus* (excluding the Hardwar Gap sample) are sister taxa, although this relationship is weakly supported. The maximum-likelihood tree is identical in topology to the parsimony tree.

Allozymic data

Parsimony analysis of allozymic data produces three equally most parsimonious trees of 105.59 steps (Fig. 6); these trees are identical for ingroup

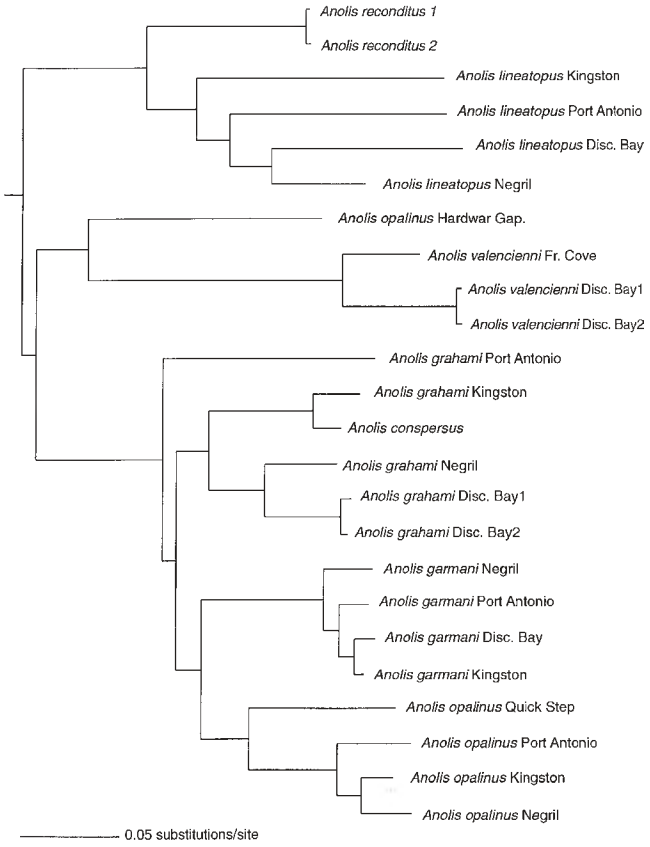

Fig. 5. A partitioning of percent nucleotide sequence divergence for mitochondrial DNA on the parsimony tree (Fig. 4) using the criterion of minimum evolution. Branch lengths are proportional to amount of sequence divergence. Average amounts of evolutionary divergence are approximately equal throughout the tree, consistent with expectations for a stochastic molecular clock.

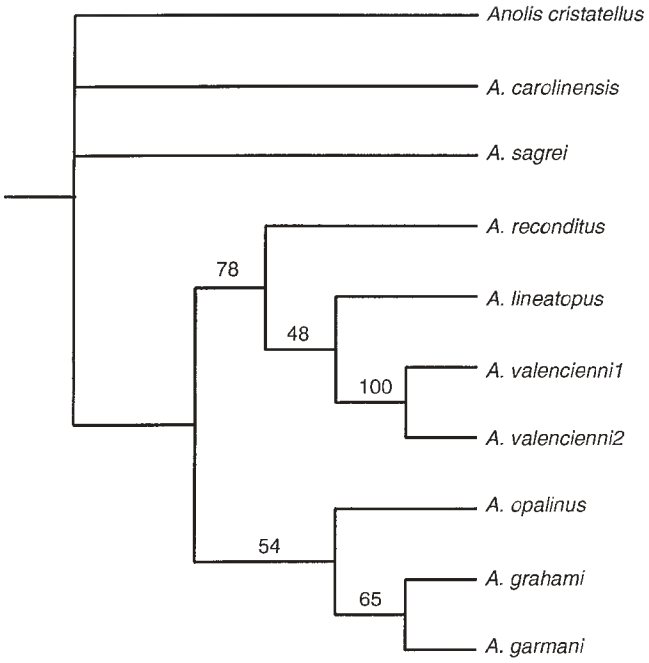

Fig. 6. A strict consensus of three equally most parsimonious trees for six species of the *A. grahami* series and three anole outgroups generated from 27 inferred allozymic loci (data from Hedges and Burnell '90) using the method of Berlocher and Swofford ('97). Numbers are bootstrap values (1,000 replicates).

relationships. The tree splits species of the *A. grahami* series into two primary clades: The first contains *A. lineatopus*, *A. reconditus*, and the two *A. valencienni* samples, whereas the second comprises *A. garmani*, *A. grahami*, and *A. opalinus*. Only two nodes appear reasonably well supported (bootstrap > 70%): 1) The node grouping *A. reconditus*, *A. lineatopus,* and *A. valencienni*, and 2) the node linking the two *A. valencienni* samples.

Combined analysis

The combined analysis, using only taxa for which both allozymic and DNA data are available, produces a single most parsimonious tree of 1,529.94 steps (Fig. 7). This tree resembles the mitochondrial DNA tree except for relationships among *A. garmani*, *A. grahami,* and *A. opalinus*. Four of seven nodes are supported by bootstrap values of 95% or larger and all by values of at least 65%. A combined analysis that includes all taxa for which DNA data are available produces a single most parsimonious tree resembling in topology the tree derived using DNA sequences alone except for relationships among *A. garmani*, *A grahami*, and *A. opalinus* (Fig. 4).

Hypothesis tests

The most parsimonious DNA tree is significantly shorter in number of DNA changes than the tree proposed by Hedges and Burnell ('90), according to the parametric-bootstrap test but not the Wilcoxon signed-ranks test (Table 1). Likewise, the DNA data reject the topology of the most parsimonious tree for the combined allozymic and DNA data (Fig. 7) using the parametric-bootstrap test but not the Wilcoxon signed-ranks test (Table 1). The most parsimonious DNA tree is significantly shorter for the DNA data than the tree based on our parsimony analysis of allozymes according to both statistical tests (Table 1). The allozymic data do not discriminate statistically between these same two topologies (n = 5, Z = 0.4, P = 0.68), nor do they reject topologies of Figure 1 (n = 4, Z = 0.13, P = 0.73), or Figure 7 (n = 5, Z = 0.14, P = 0.89). The DNA trees with *A. grahami* or *A.*

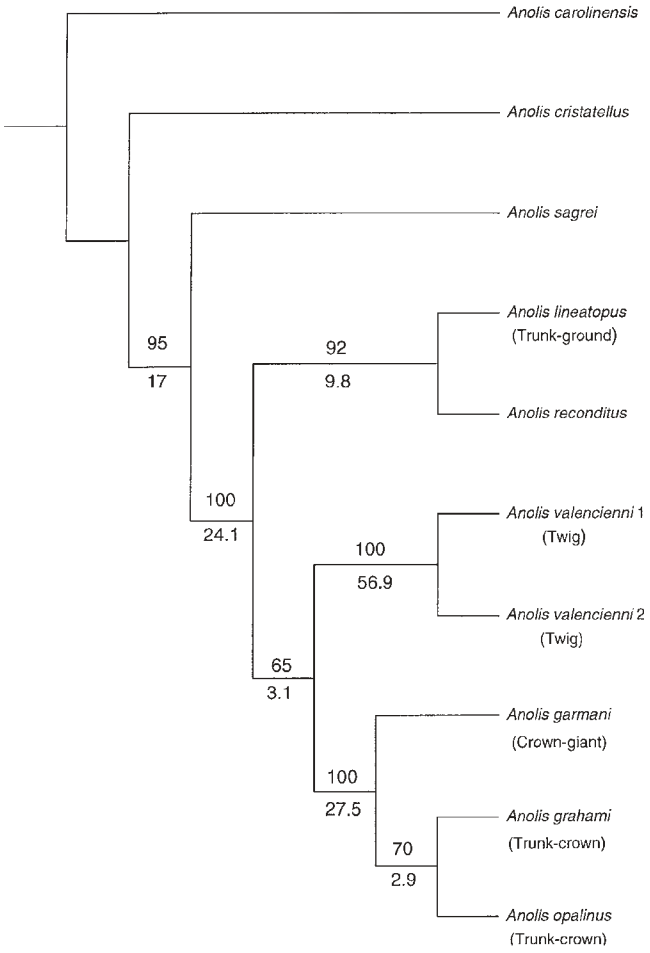

Fig. 7. The single most parsimonious tree for six species of the *A. grahami* series and three anole outgroups derived from a combined analysis of DNA and allozymic data. The length of the tree is 1,529.94 steps. Numbers above branches are bootstrap values (1,000 replicates); numbers below branches are decay-index values. Fractional decay indices result from allelic-frequency differences from allozymic data.

opalinus haplotypes constrained to be monophyletic groups are significantly less parsimonious than the shortest unconstrained DNA tree according to both tests (Table 1) using the DNA data. The shortest tree in which the *A. grahami* series is not monophyletic requires six additional steps and is not significantly longer than the most parsimonious tree for the DNA data using the Wilcoxon signed-ranks test but is significantly longer according to the parametric bootstrap (Table 1). Combined allozymic and DNA data for the 10 taxa shown in part A of Appendix 1 do not reject topological constraints of Figure 1 (n = 21, Z = 0.89, P=0.38), Figure 4 (n = 34, Z = 1.90, P = 0.06) or Figure 6 (n = 87, Z = 1.77, P=0.08) in favor of their most parsimonious tree (Fig. 7).

Tests of geographic congruence

Levels of mtDNA divergence among samples from different geographic populations of the same species are large enough to suggest that these populations represent historically distinct lineages (Table 2; Macey et al., '98a). We explore the hypothesis that vicariant events have produced similar patterns of geographic fragmentation among populations of codistributed species by examining congruence of their area cladograms. To test for congruence in phylogenetic relationships among populations of different species from the same localities, we use only those species sampled for at least three localities (*A. garmani*, *A. grahami*, *A. lineatopus*, and *A. opalinus*). The Hardwar Gap and Quick Step populations of *A. opalinus* are omitted from these tests because the other species being compared do not occur at these localities. For all pairs of species whose area cladograms show topological conflict (Fig. 4), we ask whether the conflict is statistically significant (Table 1).

Area cladograms for *Anolis grahami* and *A. opalinus* are identical for areas sampled in both species, with haplotypes from Kingston and Negril forming a clade to the exclusion of haplotypes from Port Antonio. Topologies obtained for *A. grahami* and *A. lineatopus* agree in grouping haplotypes from Discovery Bay and Negril as closest phylogenetic relatives (Fig. 8). Although the relative phylogenetic positions of Kingston and Port Antonio haplotypes are reversed in *A. lineatopus* relative to *A. grahami* and *A. opalinus*, results for *A. lineatopus* do not statistically reject the topology suggested by *A. grahami* and *A. opalinus* (Fig. 8, Table 1). Geographic relationships among haplotypes within *A. grahami* and *A. lineatopus* differ significantly from those within *A. garmani,* for which haplotypes from Discovery Bay and Kingston are closest relatives (Table 1). Sequence divergences between haplotypes from different geographic populations are greater within *A. grahami, A. lineatopus* and *A. opalinus* than within *A. garmani* (Table 2).

DISCUSSION

Our most important phylogenetic results are as follows: 1) Combined allozymic and DNA data produce a single most parsimonious tree in which *A. lineatopus* and *A. reconditus* are most closely related to one another and together form the sister group of all other species of the *A. grahami* series. Mitochondrial DNA sequences group *A.*

TABLE 1. Hypotheses tested using Wilcoxon signed-ranks tests and parametric bootstrap tests with DNA 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 hypotheses listed below can be rejected in favor of the most pasimonious tree. See Appendices 1 and 2 for precise descriptions of hypotheses tested.

		Wilcoxon signed-ranks test			Parametric bootstrap
Hypothesis	n[a]	D[b]	Z[c]	P	P
A. Hedges and Burnell ('90)	65	9	1.12	0.26	<0.01
B. Combined Analysis	38	4	0.649	0.52	0.02
C. Nonmonophyly of *A. grahami* series	57	6	0.775	0.44	0.02
D. Allozymic tree	66	30	8.09	0.0002	<0.01
E. Monophyly of *A. grahami*	39	35	5.60	<0.0001	<0.01
F. Monophyly of *A. opalinus*	94	37	3.76	0.0002	<0.01
G. Geographic constraints[d]					
A. garmani = *A. lineatopus*	64	18	2.25	0.024	<0.01
A. garmani = *A. opalinus*	56	4	0.53	0.59	0.07
A. garmani = *A. grahami*	65	13	1.61	0.107	<0.01
A. lineatopus = *A. grahami*	23	3	0.63	0.53	0.07
A. lineatopus = *A. garmani*	51	17	2.38	0.017	<0.01
A. grahami = *A. garmani*	60	30	3.69	0.0002	<0.01
A. grahami = *A. lineatopus*	35	11	1.86	0.063	<0.01
A. opalinus = *A. garmani*	21	7	1.53	0.13	0.02
A. opalinus = *A. lineatopus*	20	8	1.79	0.0736	<0.01

[a]Number of characters that differ in minimum numbers of changes on the two trees.
[b]Difference in the number of steps between the two trees.
[c]Normal approximation (Zar, '84).
[d]Haplotypes from the first species listed are constrained to the topology favored by parsimony for the second species listed (Fig. 4) for samples from Discovery Bay, Kingston, Negril, and Port Antonio; this alternative hypothesis is then tested against the most parsimonious tree for significant difference in length. *Anolis garmani, A. grahami,* and *A. lineatopus* are sampled at all four localities tested; *A. opalinus* is sampled for all localities except Discovery Bay. Except for absence of a sample from Discovery Bay, the topology obtained for *A. opalinus* matches the one for *A. grahami*. For this reason, we do not make separate comparisons for *A lineatopus* = *A. opalinus, A. grahami* = *A. opalinus*, and *A. opatinus* = *A. grahami*.

valencienni and one population of *A. opalinus* as the sister taxon to a clade containing *A. conspersus*, *A. garmani*, *A. grahami*, and *A. opalinus;* relationships among *A. garmani*, *A. grahami*, and *A. opalinus* are not well supported. 2) *Anolis opalinus* and *A. grahami* are not monophyletic for mtDNA haplotypes; *A. conspersus* is nested within *A. grahami*, and the Hardwar Gap sample of *A. opalinus* is only distantly related to other samples of *A. opalinus*. 3) Timing and sequence of fragmentation among geographic populations within *A. grahami*, *A. lineatopus*, and *A. opalinus* are roughly congruent, although fragmentation of *A. lineatopus* appears somewhat older than that of geographically codistributed populations of *A. grahami* and *A. opalinus*. Phylogeographic patterns in these species differ significantly from those of *A. garmani*, which shows considerably less haplotypic divergence among populations.

Results presented here differ in some ways from the only previous phylogenetic study of the *A. grahami* series. Hedges and Burnell ('90) suggest that *A. valencienni* is the sister taxon to other members of the series, whereas our results suggest a basal split between a clade containing *A. lineatopus* and *A. reconditus* and a clade containing *A. valencienni* and the other four species. These studies differ both in types of data (primarily allozymic versus DNA) and methods of analysis; this study uses discrete characters analyzed with parsimony and likelihood methods, whereas Hedges and Burnell ('90) use distance data analyzed with UPGMA and distance-Wagner (Farris, '72) algorithms.

We suggest that the position of *A. valencienni* in the UPGMA tree of Hedges and Burnell ('90) and the high bootstrap value at the node grouping the remaining species of the *A. grahami* series may be methodological artifacts. UPGMA analysis assumes uniform evolutionary rates among lineages (Farris, '71; Felsenstein, '82; de Queiroz and Good, '97); however, relative-rate tests (Sarich and Wilson, '67a,b) using the five outgroup species included by Hedges and Burnell ('90) and the Nei ('78) and Cavalli-Sforza and Edwards ('67) chord distances published by those authors indicate that *A. valencienni* has experienced greater allozymic divergence than the other Jamaican species from their most recent common ancestor. In addition, the distance-Wagner analysis of Hedges and Burnell ('90) provides only weak bootstrap support for the node connecting *A.*

TABLE 2. Observed percent differences between pairs of aligned DNA sequences (below) and estimated time to a common ancestor in millions of years (above). Time estimates are based on a rate of 0.65% sequence divergence per lineage per million years calibrated in recent study of lizards (Macey et al., '98a) and other vertebrates (Bermingham et al., '97; Macey et al., '98b)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1. *A. garmani DB*	–	3.5	1.2	2.2	12.2	12.4	12.4	12.4	13.4	13.5	8.8	8.8	8.7	9.2	9.5	8.7	8.9	9.4	11.3	9.2	8.9	14.3	14.2	13.2
2. *A. garmani NG*	4.5	–	3	3.2	11.9	12	12.5	12.5	12.9	13.2	9.4	9.3	9.1	9.5	9.9	8.9	8.8	9.2	11.1	8.9	8.8	13.5	13.4	12.9
3. *A. garmani KI*	1.5	3.9	–	2	12	12.2	12.1	12.1	13.2	13.1	8.5	8.4	8.3	9.2	9.5	8.5	8.7	9.3	11.1	8.8	9	14	13.8	13.2
4. *A. garmani PA*	2.9	4.1	2.6	–	11.7	12	12.3	12.3	13.2	12.8	8.8	8.7	8.7	8.9	8.9	8.6	9	9.4	11.2	9.1	9.4	13.8	13.5	12.6
5. *A. reconditus 1*	15.9	15.5	15.6	15.2	–	0.3	9.6	9.6	9.8	9.7	12.9	12.5	11.7	12.7	12.3	12.2	11.4	11.2	10.7	12.6	11.6	13.5	13.2	12.2
6. *A. reconditus 2*	16.1	15.6	15.8	15.6	0.4	–	9.7	9.7	9.9	9.7	12.9	12.5	11.8	12.8	12.5	12.2	11.6	11.4	10.8	12.7	11.8	13.6	13.4	12.5
7. *A. lineatopus DB*	17.8	17.3	17.6	17.7	14.1	14.1	–	7.6	10.6	10.5	13.8	13.8	13.2	13.9	14	13.3	13.3	12.5	13.6	13.3	13.1	14.2	14	14
8. *A. lineatopu NG*	16.1	16.3	15.7	16.0	12.5	12.6	9.9	–	8.5	9.6	13	12.8	12	13.2	13.2	12.8	12.1	11.6	12.1	12.9	12.2	13.3	13.2	12.8
9. *A. lineatopus PA*	17.4	16.8	17.1	17.1	12.8	12.9	13.8	11.1	–	10.8	13.9	13.8	13	14.1	13.8	13.5	12.4	12	12.5	12.8	12.8	14.1	14	13.8
10. *A. lineatopus KI*	17.5	17.2	17.0	16.7	12.6	12.6	13.7	12.5	14.0	–	14.2	14	13.5	13.8	13.2	13.4	13.1	12.8	12.1	13.2	13.3	13.5	13.2	13
11. *A. grahami DB 1*	11.5	12.2	11.0	11.4	16.8	16.8	17.9	16.9	18.1	18.4	–	0.6	5.1	7.3	9.7	7.3	9	9.8	11.8	8.5	9.7	13.8	13.6	12.8
12. *A. grahami DB 2*	11.4	12.1	10.9	11.3	16.3	16.3	17.9	16.6	17.9	18.2	0.8	–	4.8	7.3	9.8	7.5	8.8	9.5	11.8	8.5	9.3	13.5	13.4	12.8
13. *A. grahami NG*	11.3	11.8	10.8	11.3	15.2	15.4	17.2	15.6	16.9	17.6	6.6	6.2	–	7.6	9.2	7.2	8.2	9	11.4	8.4	8.6	13.2	13	12.8
14. *A. grahami KI*	12.0	12.3	11.9	11.6	16.5	16.6	18.1	17.2	18.3	17.9	9.5	9.5	9.9	–	9.3	2.6	9.8	10.6	11.6	9.8	10.6	14	13.8	13.8
15. *A. grahami PA*	12.3	12.9	12.3	11.6	16.0	16.3	18.2	17.1	17.9	17.2	12.6	12.8	12.0	12.1	–	9	9.7	10.2	11.8	11.2	9.8	13.5	13.3	13.3
16. *A. conspersus*	11.3	11.6	11.1	11.2	15.8	15.8	17.3	16.7	17.6	17.4	9.5	9.7	9.3	3.4	11.7	–	9.5	10.1	11.2	9.2	10.2	13.8	13.6	13.4
17. *A. opalinus KI*	11.6	11.4	11.3	11.7	14.8	15.1	17.3	15.7	16.1	17.0	11.7	11.4	10.6	12.8	12.6	12.3	–	4.1	11.3	7.9	2.6	13.1	13	12.5
18. *A. opalinus PA*	12.2	12.0	12.1	12.2	14.5	14.8	16.3	15.1	15.6	16.6	12.8	12.4	11.7	13.8	13.2	13.1	5.3	–	10.9	8.1	4.3	13.2	12.9	12.2
19. *A. opalinus HS*	14.7	14.4	14.4	14.5	13.9	14.1	17.7	15.7	16.2	15.7	15.4	15.3	14.8	15.1	15.3	14.6	14.7	14.2	–	11.8	11.4	11.5	11.5	11.8
20. *A. opalinus QS*	11.9	11.6	11.5	11.8	16.4	16.5	17.3	16.8	16.7	17.1	11.0	11.1	10.9	12.7	14.5	11.9	10.3	10.5	15.3	–	7.8	13.5	13.4	13.1
21. *A. opalinus NG*	11.6	11.5	11.7	12.2	15.1	15.4	17.0	15.9	16.6	17.3	12.6	12.1	11.2	13.8	12.8	13.2	3.4	5.6	14.8	10.1	–	13.8	13.7	13.1
22. *A. valencienni DB1*	18.6	17.6	18.2	17.9	17.5	17.7	18.5	17.3	18.3	17.5	17.9	17.6	17.1	18.2	17.6	17.9	17.0	17.1	15.0	17.5	18.0	–	0.4	5.9
23. *A. valencienni DB2*	18.4	17.4	18.0	17.6	17.2	17.4	18.2	17.1	18.2	17.1	17.7	17.4	16.9	17.9	17.3	17.7	16.9	16.8	14.9	17.4	17.8	0.5	–	6
24. *A. valencienni FC*	17.2	16.8	17.1	16.4	15.9	16.2	18.2	16.6	17.9	16.9	16.7	16.6	16.6	17.9	17.3	17.4	16.2	15.9	15.3	17.0	17.0	7.7	7.8	–

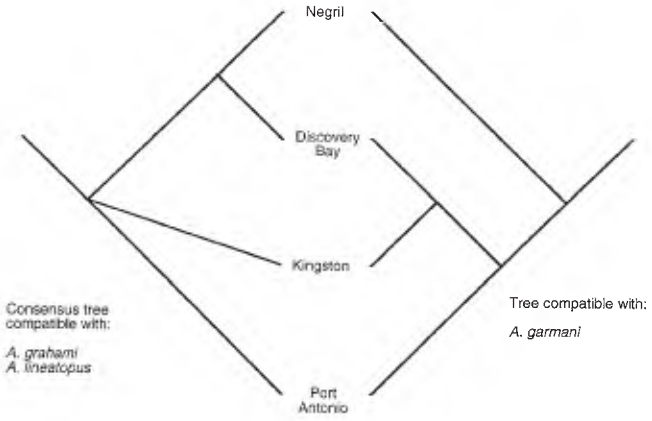

Fig. 8. Phylogenetic relationships among geographically differentiated populations within widespread species of Jamaican anoles. Results for *A. grahami* and *A. lineatopus* agree in grouping haplotypes from Discovery Bay and Negril to the exclusion of haplotypes from Kingston and Port Antonio (Fig. 4); relationships among haplotypes from geographic populations within *A. garmani* differ in grouping Discovery Bay and Kingston to the exclusion of others. Relationships among populations of *A. opalinus* (not shown), which was not sampled at Discovery Bay, agree with those found for *A. grahami* in grouping haplotypes from Kingston and Negril to the exclusion of those from Port Antonio (Fig. 4). See Table 1 for results of statistical tests of congruence among these relationships.

valencienni to the rest of the tree, suggesting that these data are equivocal regarding the phylogenetic position of *A. valencienni*.

We reanalyze Hedges and Burnell's genetic-distance data using two other additive-tree methods, that of Fitch and Margoliash ('67) as implemented in Phylip 3.5 (Felsenstein, '93) and the neighbor-joining method of Saitou and Nei ('87), as implemented in both Phylip 3.5 and PAUP*4.0b1 using various combinations of outgroup taxa with Cavalli-Sforza chord distances and Manhattan distances (from our parsimony analysis). Some of the resulting trees (not shown) place *A. valencienni* as the sister group of the remaining species, but others place it as the sister taxon to *A. lineatopus*, the species to which it exhibits the smallest genetic distance and to which it is linked in our parsimony analysis of allozymic data (Fig. 6).

Our reanalysis of allozymic data using parsimony methods yields a tree (Fig. 6) different from both that of Hedges and Burnell ('90; our Fig. 1) and the tree derived from our DNA data (Fig. 4). However, when allozymic and DNA data are combined into one analysis (Fig. 7), the resulting tree resembles the DNA tree except for relationships among *A. garmani*, *A. grahami*, and *A. opalinus*, which are not resolved with strong support in either analysis. Allozymic data do not reject the topology obtained from analysis of mitochondrial DNA sequences, and the DNA data reject the tree presented by Hedges and Burnell ('90) only using the parametric bootstrap test. The data collectively favor grouping *A. valencienni* with the *A. opalinus-A. grahami-A. garmani* clade, but further work is needed for a definitive rejection of alternative hypotheses.

Our DNA data provide estimates of divergence times for Jamaican anoles. Evolutionary rate of the mitochondrial genomic segment analyzed here has been calibrated in recent studies of lizards (Macey et al., '98a) and other vertebrates (Bermingham et al., '97; Macey et al., '98b); these studies indicate approximately 0.65% sequence divergence per lineage per million years. Using this calibration, we estimate the age of the first phylogenetic divergence within the *A. grahami* series at 13.1 million years (Table 2; range 12.8–14.4 million years). This date is nearly twice as old as Hedges and Burnell's ('90) estimate of 7 million years for the earliest divergence within the *A. grahami* series based on immunological data of Shochat and Dessauer ('81), and it approximates the estimated age of continuous emergence of Jamaica in the mid to late Miocene (Lewis and Draper, '90; Robinson, '94). Note that the estimated age of emergence of Jamaica referenced here is about half the value used by Hedges and Burnell ('90) and Schubart et al. ('98). If we apply the allozymic genetic-distance calibration for anoles (Yang et al., '74) to Hedges and Burnell's ('90) allozymic data using the topology in Fig. 5, the earliest divergence within the *A. grahami* series is estimated to be 11.3 million years, which is much closer to our DNA-based estimate. Our DNA-based estimate of the earliest phylogenetic divergence within the *A. grahami* series is approximately equivalent to the estimate of the earliest phylogenetic divergence within the frog genus *Eleutherodactylus* on Jamaica (Schubart et al., '98).

Evolution of ecomorphs

Ecomorphs of *Anolis* have evolved independently on each of the Greater Antillean islands although probably not in the same order (Losos et al., '98). Earlier phylogenetic studies indicated congruence in relationships of the different ecomorph categories on Jamaica and Puerto Rico, suggesting that these islands may have the same order of ecomorph evolution, with the lineage leading to twig anoles being the sister taxon to a clade containing all other ecomorphs (Losos, '92; Jackman et al., '97). Our phylogenetic tree challenges the hy-

pothesis that the ecomorphs evolved in the same order on these islands by calling into question the interpretation that the lineage leading to the twig ecomorph was the first lineage to diverge on Jamaica. That interpretation was based on a basal divergence of the twig-ecomorph lineage in the phylogeny of Hedges and Burnell ('90), but this lineage is not basal on our trees (Figs. 4–7). Therefore, although Greater Antillean islands share similar anole ecomorphs, our phylogeny for Jamaican anoles does not suggest the same order of ecomorph evolution on different islands.

Intraspecific divergence

An unexpected finding is the large DNA divergence among populations of *A. lineatopus* and *A. grahami* (Table 2). Paraphyly of *A. grahami* with respect to *A. conspersus* is not particularly surprising because these species have been considered closely allied (Grant, '40a,b). Four subspecies of *A. lineatopus* differ by 9.9%–14.1% in their DNA sequences, whereas *A. grahami* populations differ by 6.2%–12.8%. These values are compatible with estimates of 7.6–10.8 million years and 4.8–9.8 million years of divergent evolution between haplotypes from different populations within *A. lineatopus* and *A. grahami*, respectively (using the calibration of Macey et al., '98a,b). Figure 5 shows that parsimony reconstructs more haplotypic divergence among populations within *A. lineatopus* than between some species of the *A. grahami* series.

Although no subspecies have been described for *A. opalinus*, haplotypes from different geographic populations may be highly divergent (3.4%–15.3%), suggesting historical fragmentation of populations on the same general timescale inferred for divergences within *A. grahami* and *A. lineatopus*. The population of *A. opalinus* from Hardwar Gap may be closer phylogenetically to *A. valencienni* than to other *A. opalinus* populations.

Extensive intraspecific differentiation raises the question of whether *A. lineatopus*, *A. grahami*, and *A. opalinus* each represent a complex of species. This result may have important consequences regarding estimates of the frequency of geographic speciation on Jamaica (Losos and Schluter, 2000). The four described subspecies of *A. lineatopus* differ in dewlap and body coloration, color-changing ability, body patterning, number of subdigital lamellae, and other morphological characters (Grant, '40a; Underwood and Williams, '59; Irschick, unpublished). They differ also in thermal biology, with some subspecies being more heliophilic than others (Underwood and Williams, '59; Williams, '69; Schoener and Schoener, '71). Moreover, some evidence suggests that two subspecies may overlap geographically without intergradation in central Jamaica (Grant, '40a; Underwood and Williams, '59; Lazell, '93). Zones of intergradation have been noted, however, between several subspecies (Underwood and Williams, '59). The two subspecies of *A. grahami* are markedly different in coloration and scalation; even within *A. grahami grahami*, considerable variation is observed in color and patterning (Grant, '40a; Underwood and Williams, '59). Underwood and Williams ('59) report that in some areas the two subspecies appear to intergrade, whereas in other areas they do not; a few observations suggest sympatric occurrence of non-intergrading populations. Phenotypic distinctiveness of *A. opalinus* in the Blue Mountains (where Hardwar Gap is located) has been noted previously (Lazell, '96).

Anolis valencienni and *A. garmani* also show genetic differentiation across the island, but morphological or ecological differentiation within these species has not been noted. In *A. valencienni*, samples from Discovery Bay and Frenchman's Cove (near Port Antonio) exhibit substantially greater divergence (7.8%) than observed between comparable populations of *A. garmani* (2.8%) but not as great as between comparable populations of *A. lineatopus* (13.8%). Unfortunately, we have no samples of *A. valencienni* from Kingston and Negril. Intraspecific differentiation within *A. garmani* appears more recent (maximum of 3.2 million years) than that of the other species (maxima of 7.5 to 9.7 million years) and displays different geographic patterns. These dates suggest at least three phases of geographic differentiation of anoles on Jamaica (Fig. 8). In the first phase, *A. lineatopus* spread across the island and became geographically fragmented. In the second phase, *A. grahami* and *A. opalinus* spread across the island and became geographically subdivided with perhaps the same historical events influencing geographic fragmentation in these two species. In the third phase, *A. garmani* spread across the island and became geographically subdivided. Further sampling is needed to determine whether geographic fragmentation among codistributed populations of *A. valencienni* matches any of these three patterns.

Recent studies of genetic differentiation in several Lesser Antillean anole species also reveal extensive intraspecific differentiation. *Anolis marmoratus* (Guadeloupe) and *A. oculatus* (Do-

minica) both show extensive geographic variation in morphology, so much that 12 subspecies of *A. marmoratus* have been described in the Guadeloupean archipelago (Lazell, '64, '72). Phylogeographic studies using mitochondrial DNA reveal extensive geographic differentiation within both species, comparable to that reported here for *A. lineatopus* and *A. grahami* (Malhotra and Thorpe, 2000; Schneider, '96).

Like Jamaica, both Guadeloupe and Dominica are topographically diverse islands that exhibit considerable geographic variation in rainfall. Malhotra and Thorpe ('91, '94, '97a,b, 2000) have shown that geographic variation in morphology within *A. marmoratus* and *A. oculatus* correlates with climate, elevation, and habitat type. A similar relationship between climate and coloration, in which populations in wetter areas tend to be greener and those in drier areas are more brown, occurs in *A. lineatopus* and *A. grahami* (Underwood and Williams, '59; Lazell, '96); further work should investigate whether scalation and morphometric variation in these species relates to environmental variation as in the two Lesser Antillean species.

Most research on anole radiation has focused on adaptation and diversification among recognized species and major clades. Our findings of substantial geographic fragmentation within recognized Jamaican species, coupled with similar recent findings on Lesser Antillean species and Amazonian species (Glor et al., 2001), suggest another important dimension of diversification, and the possibility that species diversity is considerably underestimated. Unlike older divergences, which demonstrate ecomorphological divergence as a result of interspecific competition (Williams, '72, '83; Losos, '92, '95), initial stages of differentiation may result primarily from geographic isolation of populations (Lazell, '96).

ACKNOWLEDGMENTS

We thank A. Donaldson and the Natural Resources Conservation Department, Jamaica, for permission to collect specimens and L. Fleishman, M. Haley, D. Reddie, and the Discovery Bay Marine Laboratory for assistance. We thank M. Leal, G. Mayer, and L. Vitt for tissue samples. S.B. Hedges provided corrections to Hedges and Burnell's ('90) data set, and J. Wilgenbusch helped with allozyme-frequency analysis. G. Draper provided information on the geological history of Jamaica. J. R. Macey and anonymous reviewers provided helpful comments on earlier drafts of the manuscript.

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APPENDIX 1

Phylogenetic constraints used to find the shortest trees compatible with various hypotheses tested using the DNA, allozymic, and combined data sets. These hypotheses are used in pairwise tests with topologies favored by the respective data sets to ask whether the data statistically reject them in favor of the overall most parsimonious tree(s).

A. Constraints used in analyses of allozymic and combined data for ten samples that have both allozymic and DNA data available. Constraints 1, 3, and 4 are used also as backbone constraints in analyses of the DNA data alone (all samples). Samples are numbered as follows: 1 = *Anolis garmani* Discovery Bay, 2 = *Anolis grahami* Discovery Bay 1, 3 = *Anolis lineatopus* Discovery Bay, 4 = *Anolis opalinus* Quick Step, 5 = *Anolis reconditus* 1, 6 = *Anolis sagrei,* 7 = *Anolis valencienni* Discovery Bay 1, 8 = *Anolis valencienni* Frenchman's Cove, 9 = *Anolis cristatellus,* 10 = *Anolis carolinensis.*

Hedges and Burnell ('90) = ((((1,(2,4)),(3,5)),(7,8)),6,9,10)

All DNA data = (((((1,4),2),(7,8)),(3,5)),9,10,6)

Allozymic tree = ((((1,2),4),((3,(7,8)),5)),9,10,6)

Combined analysis = ((((1,(2,4)),(7,8)),(3,5)),9,10,6)

B. Constraints used in analyses of the complete DNA dataset (Fig. 4, Table1). Samples are numbered as follows: 1 = *Anolis carolinensis,* 2 = *Anolis cristatellus,* 3 = *Anolis chrysolepis,* 4 = *Anolis carpenteri,* 5 = *Anolis lemurinus,* 6 = *Anolis fuscoauratus,* 7 = *Anolis trachyderma*, 8 = *Anolis biporcatus,* 9 = *Anolis lineatus,* 10 = *Anolis ortoni,* 11 = *Anolis garmani* Discovery Bay, 12 = *Anolis garmani* Negril, 13 *Anolis garmani* Kingston, 14 = *Anolis garmani* Port Antonio, 15 = *Anolis reconditus* 1, 16 = *Anolis reconditus* 2, 17 = *Anolis lineatopus* Discovery Bay, 18 = *Anolis lineatopus* Negril, 19 = *Anolis lineatopus* Port Antonio, 20 = *Anolis lineatopus* Kingston, 21 = *Anolis grahami* Discovery Bay 1, 22 = *Anolis grahami* Discovery Bay 2, 23 = *Anolis grahami* Negril, 24 = *Anolis grahami* Kingston, 25 = *Anolis grahami* Port Antonio, 26 = *Anolis conspersus,* 27 = *Anolis opalinus* Kingston, 28 = *Anolis opalinus* Port Antonio, 29 = *Anolis opalinus* Blue Mountains, 30 = *Anolis opalinus* Quick Step, 31 = *Anolis opalinus* Negril, 32 = *Anolis valencienni* Discovery Bay 1, 33 = *Anolis valencienni* Discovery Bay 2, 34 = *Anolis valencienni* Frenchman's Cove, 35 = *Anolis allogus,* 36 = *Anolis imias,* 37 = *Anolis sagrei,* 38 = *Anolis ahli*, 39 = *Anolis ophiolepis.*

Nonmonophyly of *A. grahami* series (employed as a reverse constraint) = (35,36,37,38,39,10,9,8,7,6,5,4,3,2,1,(11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34))

A. grahami (species) monophyletic = (1,2,35,38,36,37,39,3,9,8,4,5,7,6,10,15,16,17,18,19,20,29,32,33,34,11,13,14,12,27, 31,28, 30,26,(25,21,22,23,24))

A. opalinus monophyletic = (1,2,35,38,36,37,39,3,9,8,4,5,7,6,10,15,16,17,18,19,20,32,33,34,11,13,14,12,(27,29,31,28,30),26,25,21,22,23,24)

Geographic Constraints (9) All employed as backbone constraints.

A. garmani = *A. lineatopus* = (1,2,3,4,5,6,7,8,9,10,(13,(14,(11,12))),15,16,20,17,18,19,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39)

A. garmani = *A. opalinus* = (1,2,3,4,5,6,7,8,9,10,11,((12,13),14),15,16,20,17,18,19,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39)

A. garmani = *A. grahami* = (1,2,3,4,5,6,7,8,9,10,((13,(11,12)),14),15,16,20,17,18,19,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39)

A. lineatopus = *A. grahami* = (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,((20,(17,18)),19),21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39)

A. lineatopus = *A. garmani* = (29,26,16,15,2,3,9,8,4,5,7,6,10,32,33,34,11,13,12,14,27,31,28,30,23,25,21,22,24, (18,(19,(17,20))),35,38,36,37,39,1)

A. grahami = *A. garmani* = (29,26,16,15,2,3,9,8,4,5,7,6,10,32,33,34,11,13,12,14,27,31,28,30,(23,(25,((21,22),24))),17,18,19,20,35,38,36,37,39,1)

A. grahami = *A. lineatopus* = (1,2,35,38,36,37,39,3,9,8,4,5,7,6,10,15,16,17,18,19,20,29,32,33,34,11,13,14,12,27,31,28,30,26,(24,(25,((22,21),23))))

A. opalinus = *A. garmani* = (1,2,35,38,36,37,39,3,9,8,4,5,7,6,10,15,16,17,18,19,20,32,33,34,11,13,14,12,((27, 28),31),26,25,21,22,23,24,30,29)

A. opalinus = *A. lineatopus* = (1,2,35,38,36,37,39,3,9,8,4,5,7,6,10,15,16,17,18,19,20,32,33,34,11,13,14,12, (((31,28),27)26,25,21,22,23,24,30,29)

APPENDIX 2

Trees used in simulations for parametric-bootstrap tests. Branch lengths follow taxon numbers (terminal branches) or parentheses (internal branches) and are expected numbers of substitutions per base pair. Taxa are numbered as described in Appendix 1.

A. Hedges and Burnell's ('90) hypothesis: (1:0.000000,(2:0.114811,(((((3:0.102591,5:0.080927):0.056219,4:0.087957):0.058452, ((6:0.162904,7:0.125517):0.025675,((((((8:0.012312,10:0.004766):0.008029,11:0.013716):0.009846,9:0.018734):0.043348,((((18:0.004489,19:0.003369):0.033195,20:0.032129):0.021510,(21:0.021294,22:0.015560):0.043651):0.020790,((23:0.024001,24:0.032451):0.034523,26:0.057500):0.018162):0.010385):0.048820,((12:0.001712,13:0.002598):0.056887,(((14:0.070542,15:0.038980):0.021463,16:0.076648):0.015783,17:0.084784):0.021344):0.036784):0.008240,(25:0.081350,((27:0.002164,28:0.002784):0.045171,29:0.034139):0.072234):0.022624):0.016295):0.011651):0.032811,((30:0.034142,32:0.051169):0.103139,(31:0.058449,33:0.071126):0.076194):0.025643):0.042017,34:0.136480):0.037397):0.136926)

B. Combined Analysis: (1:0.000000,(2:0.115374,((((((3:0.103472,5:0.079712):0.055309,4:0.088650):0.056631,(6:0.163525,7:0.126006):0.017498):0.016399,((((((8:0.012308,10:0.004770):0.008115,11:0.013634):0.009841,9:0.018782):0.044806,((((18:0.004495,19:0.003363):0.032927,20:0.032380):0.021845,(21:0.021237,22:0.015606):0.043416):0.019783,((23:0.024063,24:0.032384):0.034007,26:0.058053):0.019282):0.009045):0.044960,(25:0.082277,((27:0.002169,28:0.002778):0.046577,29:0.032660):0.071915):0.016652):0.018326,((12:0.001746,13:0.002563):0.056730,(((14:0.069932,15:0.039573):0.021884,16:0.076844):0.014866,17:0.085203):0.022224):0.032159):0.014601):0.033152,((30:0.035540,32:0.049716):0.102573,(31:0.058635,33:0.071057):0.077320):0.026044):0.043761,34:0.134807):0.038022): 0.136222);

C. Nonmonophyly of the *A. grahami* series: (1:0.000000,(2:0.183546,(((((3:0.139073,5:0.116141):0.082218,4:0.128765):0.096468,(((6:0.288160,7:0.210454):0.027070,((12:0.001715,13:0.002780):0.077672,(((14:0.089100,15:0.046417):0.023153,16:0.099037):0.014342,17:0.114758):0.025338):0.043569):0.002822,((((((8:0.012984,10:0.005054):0.007963,11:0.015124):0.009013,9:0.022379):0.058939,((23:0.024793,24:0.037984):0.042217,26:0.068776):0.019977):0.011009,(((18:0.004530,19:0.003478):0.035945,20:0.036978):0.024463,(21:0.022730,22:0.016816):0.053074):0.016734):0.059697,(25:0.113227,((27:0.002287,28:0.002853):0.055935,29:0.035353):0.106957):0.018814):0.025185):0.015489):0.041015,((30:0.039302,32:0.060485):0.175315,(31:0.071544,33:0.095494):0.136287):0.032857):0.069274,34:0.221470):0.050965):0.237952)

D. Tree obtained from analysis of allozymic data: (1:0.000000,(2:0.115378,((((((3:0.103762,5:0.079421):0.055669,4:0.088416):0.055852,(6:0.162991,7:0.127170):0.017215):0.015345,((((((8:0.012308,10:0.004771):0.007929,11:0.013798):0.009535,9:0.019119):0.046980,(((18:0.004491,19:0.003367):0.032667,20:0.032511):0.022385,(21:0.021299,22:0.015463):0.041923):0.020675):0.008505,((23:0.024384,

24:0.032024):0.034854,26:0.056402):0.017413):0.058561,((12:0.001612,13:0.002689):0.073733,((((14:0.070128,15:0.039461):0.018295,16:0.080917):0.018162,17:0.083479):0.033220,(25:0.082621,((27:0.002161,28:0.002787):0.044725,29:0.034612):0.071390):0.033548):0.000539):0.015634):0.017015):0.034038,((30:0.035531,32:0.049726):0.102185,(31:0.058888,33:0.070808):0.077732):0.027709):0.042317,34:0.135246):0.037932):0.135989);

E. Monophyly of *A. grahami*: (1:0.000000,(2:0.184680,((((((3:0.141451,5:0.115240):0.085591,4:0.126419):0.091142,(6:0.287070,7:0.208988):0.011523):0.017604,(((((((8:0.013016,10:0.005096):0.008178,11:0.015024):0.009931,9:0.021311):0.057603,((23:0.024934,24:0.038151):0.042719,26:0.069179):0.021810):0.010350,((((18:0.004412,19:0.003630):0.037030,20:0.036459):0.066643,21:0.024890):0.0000000106,22:0.019705):0.063551):0.057858,(25:0.114157,((27:0.002287,28:0.002874):0.056193,29:0.035395):0.106198):0.018262):0.020032,((12:0.001728,13:0.002788):0.075800,(((14:0.090215,15:0.046568):0.021669,16:0.100419):0.015962,17:0.114624):0.025899):0.042883):0.015439):0.043578,((30:0.040711,32:0.059512):0.176554,(31:0.071767,33:0.095826):0.136960):0.032932):0.071957,34:0.221706):0.052631):0.238704);

F. Monophyly of *A. opalinus*:

(1:0.000000,(2:0.189360,(((((((3:0.146203,5:0.116268):0.086638,4:0.133444):0.084758,6:0.297046):0.012668,7:0.226267):0.017347,(((((((8:0.013258,10:0.005205):0.008103,11:0.015516):0.008888,9:0.023328):0.065010,(((18:0.004670,19:0.003524):0.036807,20:0.037981):0.024900,(21:0.023044,22:0.017452):0.054216):0.020250):0.010494,(((23:0.026962,24:0.037276):0.041285,26:0.071467):0.028151,25:0.174434):0.000000003324):0.054539,((27:0.002323,28:0.002941):0.059439,29:0.034564):0.122402):0.019587, (12:0.001781,13:0.002828):0.082471,(((14:0.091230,15:0.048915):0.022813,17:0.124731):0.006329,16:0.103677):0.028834):0.042758):0.014888):0.044639,((30:0.041880,32:0.060714):0.182369,(31:0.073487,33:0.098717):0.142771):0.033364):0.072783,34:0.229648):0.052938):0.247837);

G. Closest (in parsimony steps) geographic congruence (*A. garmani* = *A. opalinus*): (1:0.000000,(2:0.184831.((((((3:0.141427, 5:0.115890):0.085446,4:0.128073):0.090987,(6:0.287735,7:0.210220):0.011172):0.017508,((((((8:0.013858,(10:0.005924,11:0.022213):0.000000):0.012752,9:0.025158):0.059316,((23:0.024827,24:0.038465):0.043226,26:0.068731):0.020139):0.011125,(((18:0.004556,19:0.003507):0.036325,20:0.037046):0.024176,(21:0.022944,22:0.016897):0.054227):0.016048):0.062238,(25:0.112273,((27:0.002299,28:0.002877):0.056168,29:0.035890):0.109720):0.019085):0.020176,((12:0.001728,13:0.002799):0.076136,(((14:0.090472,15:0.046498):0.021175,16:0.101282):0.015443,17:0.116749):0.026469):0.043771):0.014937):0.043664,((30:0.041377,32:0.059168):0.177099,(31:0.072190,33:0.096142):0.138445):0.033013):0.072182,34:0.222542):0.052352):0.240568);