A molecular phylogenetic analysis of diversification in Amazonian *Anolis* lizards

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Abstract

We present a mitochondrial DNA (mtDNA) haplotype phylogeny for Amazonian *Anolis* lizards, including geographical sampling within four species distributed across the Amazon basin (*A. fuscoauratus*, *A. nitens*, *A. ortonii* and *A. punctatus*). Approximately 1500 bp of mtDNA encoding ND2, COI and four transfer RNAs (tRNAs) are reported for 39 specimens representing four to five populations of each widespread species, plus eight outgroups. These new sequences are aligned with eight previously published sequences, yielding 914 variable characters and 780 parsimony-informative characters. Phylogenetic analyses using maximum parsimony and maximum likelihood reject the hypothesis that Amazonian anoles form a monophyletic group excluding Central American and Caribbean anoles, and suggest multiple faunal exchanges among these regions. Haplotype divergence among geographical populations of *A. nitens*, whose variation was influential in formulating the Pleistocene refuge hypothesis of Amazonian speciation, is very large (13–22% sequence difference), suggesting that these populations separated well before the Pleistocene. Haplotype divergences among geographical populations of *A. fuscoauratus* (3–4%), *A. punctatus* (4–9%) and *A. ortonii* (6–8%) also indicate pre-Pleistocene differentiation within each species, but temporally incongruent patterns among species.

Keywords: Amazon, *Anolis*, biogeography, phylogeography, Pleistocene, refuge hypothesis

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Introduction

Hypotheses proposed to explain the remarkable diversification of tropical rainforest faunas often invoke Pleistocene fragmentation of forests as a causal factor initiating evolutionary divergence of populations (Haffer 1969, 1997; Vanzolini & Williams 1970, 1981; Vanzolini 1973). Recent molecular phylogenetic studies challenge this notion by indicating that most divergences between sister species of tropical rainforest vertebrates considerably predate the Pleistocene (reviewed by Moritz *et al.* 2000). This observation also refutes arguments that major rivers in their current locations were responsible for initiating speciation among terrestrial Amazonian vertebrates (Moritz *et al.* 2000). Molecular phylogenetic studies of terrestrial rainforest vertebrates are scarce (Moritz *et al.* 2000), however, and none examine the squamate taxa initially used to formulate

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Pleistocene refuge hypotheses for Amazonia (Vanzolini & Williams 1970).

Molecular studies of Amazonian terrestrial vertebrates show that geographically codistributed taxa often do not demonstrate congruent area relationships (Patton *et al.* 1996, 2000; Patton & da Silva 1998; Cropp *et al.* 1999; Johnson *et al.* 1999; Ditchfield 2000; Matocq *et al.* 2000). Different taxa appear to have entered the Amazon region at different times and show different spatial and temporal patterns of fragmentation, a phenomenon recognized as the 'deep history' problem of continental biogeography (Cracraft 1988).

Vanzolini & Williams (1970) propose that *Anolis* lizards entered Amazonia as many as four separate times before the Pleistocene, and that geographical differentiation among populations within each species results largely from Pleistocene vicariance. Four anole species (*A. fuscoauratus*, *A. nitens*, *A. ortonii*, *A. punctatus*) occupy the entire Amazon basin, and numerous others have more localized distributions there (Vanzolini & Williams 1970; Avila-Pires 1995). *A. punctatus* is considered to have an ancient origin in South

America, whereas the other widespread species are thought to have entered South America from Central America in separate pre-Pleistocene invasions (Vanzolini & Williams 1970). Citing its morphological diversity, Vanzolini & Williams (1970) suggest that *A. nitens* was the first of the latter species to enter South America, followed by *A. ortonii* and then *A. fuscoauratus*, which is barely distinguishable morphologically from its Central American counterpart, *A. limifrons* (Vanzolini & Williams 1970).

We present a phylogenetic analysis of mitochondrial DNA (mtDNA) haplotypes sampled from multiple geographical populations of each widespread Amazonian anole species and include representatives of two additional Amazonian species (*A. transversalis* and *A. trachyderma*) and 14 non-Amazonian species (*A. aeneus*, *A. agassizi*, *A. carpenteri*, *A. grahami*, *A. humilis*, *A. lemurinus*, *A. limifrons*, *A. lineatopus*, *A. lineatus*, *A. mestrei*, *A. microtus*, *A. sagrei*, *A. woodi*, *Phenacosaurus nicefori*) to clarify phylogenetic affinities and historical biogeography of Amazonian anoles.

Materials and methods

DNA extraction and sequencing

DNA extraction and amplification were conducted as described by Jackman *et al.* (1999). Sequencing reactions were run initially using Promega fmol DNA sequencing systems as described by Macey *et al.* (2000). Additional reactions were run with Big-Dye Terminator Ready-Reaction Kit (Perkin-Elmer) on an ABI™ (PE Applied Biosystems, Inc.) 373A automated DNA sequencer.

We sequenced ≈ 1500 bp of mtDNA, including complete sequence for genes encoding ND2, tRNA^{Trp}, tRNA^{Ala}, tRNA^{Asn}, tRNA^{Cys}, tRNA^{Tyr}, origin of light-strand replication, and part of COI. Most sequence (≈ 70%) was obtained from both strands. Sequences were aligned manually. Alignment of transfer RNA (tRNA) genes utilized secondary structural models (Kumazawa & Nishida 1993; Macey *et al.* 1997). The origin of light-strand replication, several length-variable tRNA loops (tRNA^{Trp}, tRNA^{Cys}, and tRNA^{Tyr} D loops; tRNA^{Trp} and tRNA^{Cys} T loops), and length-variable regions between tRNA genes (tRNA^{Trp}–tRNA^{Ala}, tRNA^{Ala}–tRNA^{Asn}, tRNA^{Cys}–tRNA^{Tyr}) were excluded from phylogenetic analyses because of ambiguous alignments, leaving 1412 aligned base pairs.

Sequences were obtained for four to five geographically separated populations of *Anolis fuscoauratus*, *A. nitens*, *A. ortonii* and *A. punctatus*, and 10 outgroup taxa (Appendix I, Fig. 1). One to three individuals were sampled per population (Appendix I). *Polychrus acutirostris* served as an outgroup for all *Anolis* (Jackman *et al.* 1999). Additional outgroups included five Caribbean species, four of which (*A. grahami*, *A. lineatopus*, *A. mestrei*, *A. sagrei*) have been grouped taxonomically with *A. fuscoauratus*, *A. nitens* and
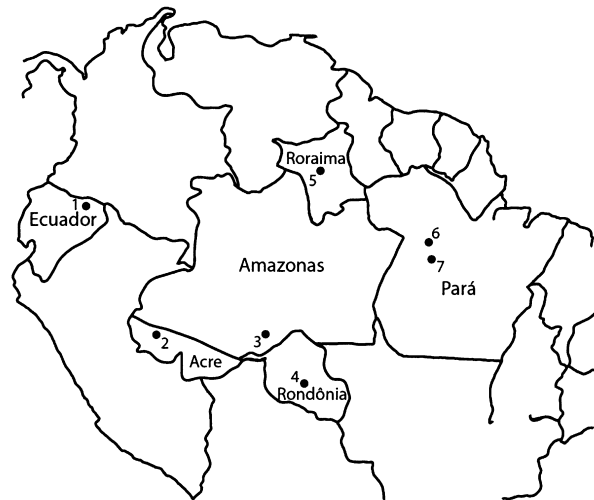

Fig. 1 Map of localities sampled. Circles mark collection localities for anoles used in this study (as numbered in Appendix I). Bold labels represent countries (Ecuador) and Brazilian states (Acre, Amazonas, Pará, Roraima, Rondônia) from which anoles were sampled.

A. ortonii (genus *Norops* of Guyer & Savage 1986; Savage & Guyer 1989), and one of which (*A. aeneus*) has been grouped taxonomically with *A. punctatus* (genus *Dactyloa* of Guyer & Savage 1986; Savage & Guyer 1989). All available mainland *Norops* (*A. carpenteri*, *A. humilis*, *A. lemurinus*, *A. lineatus*, *A. trachyderma*, *A. woodi*) and *Dactyloa* (*Phenacosaurus nicefori*, *A. agassizi*, *A. microtus*, *A. transversalis*) are sampled. Museum and GenBank accession numbers and localities for specimens examined are in the Appendix. Animals were treated according to federal, state and university regulations (Animal Care Assurance 73-R-100, approved 8 November 1994).

Sequence and phylogenetic analysis

PAUP* beta version 4.0b4a (Swofford 2000) was used to generate phylogenetic trees under parsimony and likelihood criteria. Parsimony trees were generated using 100 heuristic searches with random addition of sequences. The HKY model (Hasegawa *et al.* 1985) incorporating rate variation (Γ) and invariable sites (I) was utilized for all likelihood analyses based on hierarchical hypothesis testing of alternative models implemented with MODELTEST 3.0 (Posada & Crandall 1998). The transition/transversion ratio, gamma shape parameter, and proportion of invariant sites were estimated by maximum likelihood from the maximum-parsimony tree.

For the parsimony tree, bootstrap resampling and decay indices ('branch support' of Bremer 1994) were used to assess support for individual nodes. Bootstrapping was conducted in PAUP* using 1000 bootstrap replicates with 25 random additions per replicate. Decay indices were calculated with TREEROT version 2 (Sorenson 1999). Where appropriate,

support for particular groupings was tested with the Wilcoxon signed-ranks test as applied by Templeton (1983) but with two-tailed probability values (Felsenstein 1985).

Uncorrected per cent sequence differences and maximum-likelihood distances under the HKY model were generated in PAUP*. The maximum-likelihood distances compensate substitutional saturation. We also examined saturation heuristically by plotting HKY corrected distances against number of substitutions for the entire sequence and two subsets of this sequence as done by Jackman *et al.* (1999).

Compatibility of our data with a stochastic clock-like model of evolution was tested using the branch-length test of Takezaki *et al.* (1995), which permits identification and exclusion of lineages having abnormally high or low evolutionary rates. Approximate divergence dates were obtained using the calibration 0.65% divergence per lineage per million years obtained from agamid lizards for the same mitochondrial genes (Macey *et al.* 1998).

Results

Thirty-nine new mtDNA sequences are reported and aligned with eight previously reported sequences giving an alignment with 1522 sites. Absence of premature stop codons, functional stability of tRNA genes, and strong bias against guanine in the light strand all confirm that new sequences represent authentic mtDNA (Zhang & Hewitt 1996). Phylogenetic analyses exclude 110 sites having uncertain alignment, leaving 1412 included characters, 914 of which are variable and 780 of which are parsimony informative. Parsimony analysis produces 18 equally most parsimonious trees of 4752 steps (Fig. 2). Parameters used for likelihood analysis are: transition/transversion ratio = 2.67; gamma shape parameter = 0.88; proportion of invariable sites = 0.26. Maximum-likelihood analysis produced a single tree with a score of 21231.73; it differs in topology from the maximum-parsimony tree only by grouping *Anolis aeneus* and *Phenacosaurus nicefori* as the sister taxon to the clade containing *A. punctatus* and *A. transversalis* (Fig. 2).

Two previously proposed clades of mainland anoles (*Norops* and *Dactyloa* of Savage & Guyer 1989) each appear as monophyletic groups in both parsimony and likelihood trees (Fig. 2) provided that *Phenacosaurus* is included in *Dactyloa*. Monophyly of *Norops* is well supported by bootstrap and decay-index values, whereas monophyly of *Dactyloa* is moderately supported (Fig. 2). The conservative Templeton test, however, does not reject nonmonophyly for either group (*Dactyloa P* < 0.41; *Norops P* < 0.49).

Within *Dactyloa*, Amazonian species *A. punctatus* and *A. transversalis* are grouped with strong support (Fig. 2). Within *Norops*, both likelihood and parsimony analyses group Cuban anoles (*A. mestrei* and *A. sagrei*) as a deep branch whose exact relationship to other groups is not well resolved (Fig. 2). A basal polytomy occurs in the bootstrap tree for remaining *Norops*, including taxa from Jamaica, Central America and South America. Jamaican taxa (*A. grahami* and *A. lineatopus*) form another basal branch whose exact phylogenetic placement is uncertain in the parsimony and likelihood analyses (Fig. 2). Within mainland *Norops*, *A. fuscoauratus*, *A. ortonii* and numerous other Central and South American taxa form a well-supported clade. Haplotypes from each of the widespread Amazonian species form monophyletic groups and, except for *A. nitens*, each group is well supported. Neither the *A. auratus* nor the *A. fuscoauratus* series of Savage & Guyer (1989) form monophyletic groups in the parsimony and likelihood trees; monophyly of the *A. auratus* series (*A. lemurinus*, *A. humilis*, *A. lineatus* and *A. nitens*) is rejected (*P* < 0.0012), whereas monophyly of the *A. fuscoauratus* series (*A. carpenteri*, *A. limifrons*, *A. trachyderma*, *A. fuscoauratus*, *A. ortonii*) is not (*P* < 0.083). Removal of *A. ortonii* from the *A. fuscoauratus* group as recommended by Köhler (1996) does not render this group monophyletic. Furthermore, Amazonian *Norops* (*A. fuscoauratus*, *A. nitens*, *A. ortonii* and *A. trachyderma*) do not form a monophyletic group with respect to Central American and trans-Andean *Norops* (*A. carpenteri*, *A. lemurinus*, *A. limifrons*, *A. humilis*, *A. woodi*); monophyly of Amazonian *Norops* is strongly rejected (Templeton test, *P* < 0.0001).

Within species, haplotypes from the same population always form strongly supported clades; however, few interpopulational groupings of haplotypes are well supported except within *A. nitens*. Within *A. nitens*, a very deep split occurs between haplotypes from north (Roraima and Ecuador) and south (Amazonas and Acre) of the Amazon River.

Genetic distances

Pairwise differences between haplotypes within populations of each species (0–3%) are always lower than interpopulation differences, with some individuals from the same population sharing haplotypes (Table 1). Whereas *A. fuscoauratus* (3–4%), *A. punctatus* (4–9%) and *A. ortonii* (6–8%) exhibit moderate levels of interpopulation divergence in haplotype sequence, *A. nitens* demonstrates extremely high levels of interpopulational divergence (13–22%); haplotypes from north (Roraima and Ecuador) vs. south (Acre and Amazonas) of the Amazon River differ by over 20% uncorrected sequence difference, comparable with interspecific differences. Even within northern and southern haplotype groupings of *A. nitens*, sequence divergence exceeds 10% between populations. Most interspecific contrasts of haplotypes exceed 20% divergence.

Maximum-likelihood (HKY) distances are consistently several percentage points higher for contrasts > 10% uncorrected difference, suggesting some substitutional saturation (Table 1). A plot of HKY-corrected distance vs. number of substitutions (not shown) suggests that
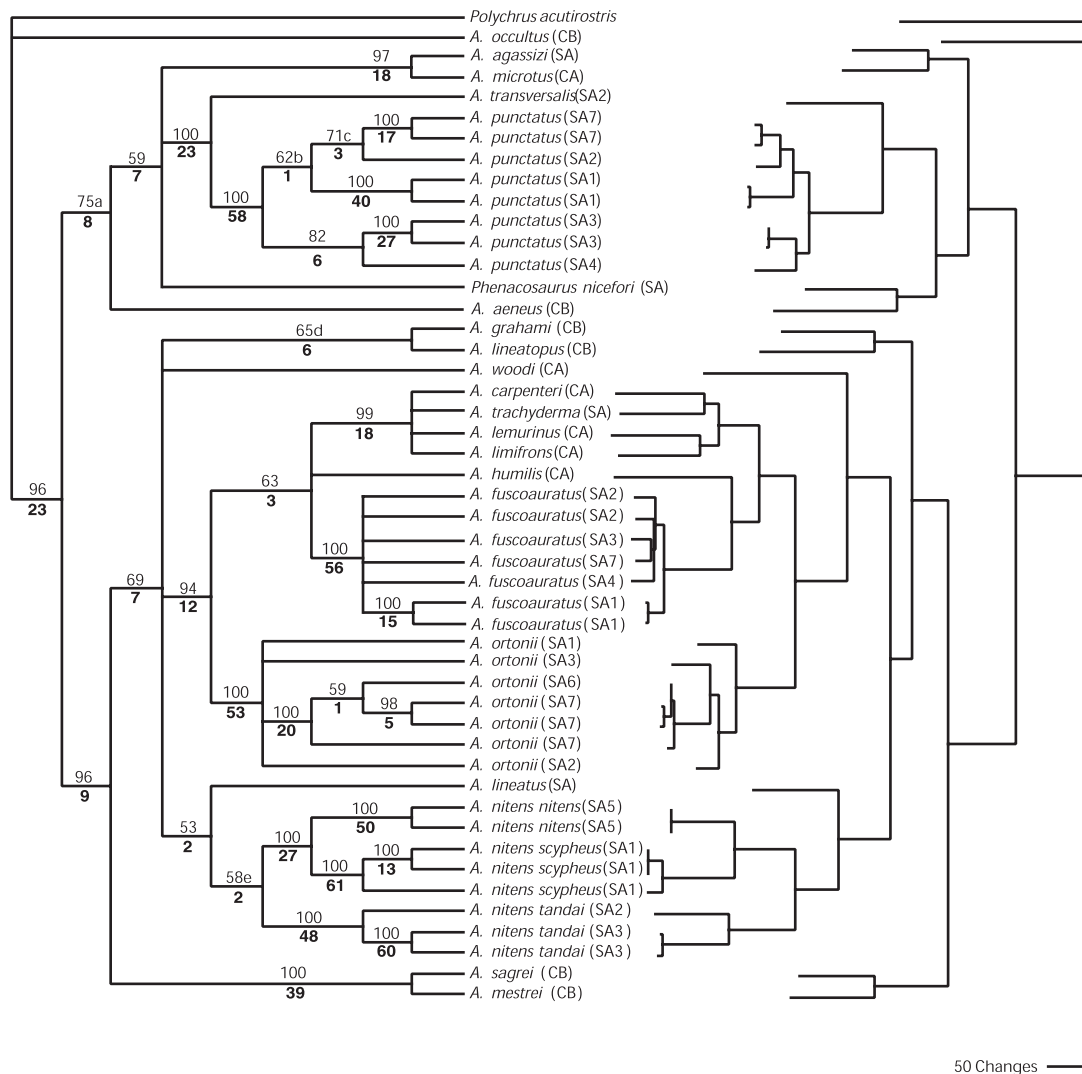
Fig. 2 Maximum parsimony bootstrap tree (left) and maximum-likelihood tree (right). Bootstrap values are given above branches and decay indices below branches on the parsimony tree; branches with bootstrap values < 50% are collapsed. Letters (a–e) mark five branches whose support (bootstrap, decay index) increases to the following values when the analysis eliminates third-position transitions: a (92, 10), b (89, 3), c (90, 4), d (87, 10), e (82, 6). Branch lengths on the maximum-likelihood tree are proportional to amount of evolutionary change.

third-position transitions become noticeably saturated above 10% sequence divergence.

A parsimony analysis with third-position transitions excluded shows stronger support for five nodes than the parsimony analysis including this variation (Fig. 2). Support for monophyly of *Dactyloa* and *A. nitens* increases (Fig. 2); however, the hypothesis of nonmonophyly is still not rejected in either case (*Dactyloa P* < 0.13; *A. nitens*: *P* < 0.40).

The branch-length test (Takezaki *et al.* 1995) indicates that our results generally conform to a stochastic molecular clock, with a few anomalies. Lineages leading to outgroup taxa are generally shorter than others, as is the common ancestral lineage of *A. punctatus* and *A. transversalis*. Ages for nodes in a haplotype tree can be estimated by averaging

amounts of divergence occurring between their paired lineages. Uncorrected differences are used for all age estimates with the expectation that ages are underestimated when differences exceed 10%.

Maximum sequence differences within a species may be used to obtain minimum estimates of its age of origin provided that haplotypes coalesce within the species. Haplotype divergences within *A. punctatus* and *A. ortonii* suggest that these species are at least 6–7 Myr old. The minimum age of *A. fuscoauratus* is somewhat more recent at 3 Myr. *Anolis nitens* has, by far, the oldest minimum age, > 15 Myr. Almost all interspecific differences exceed 20%, suggesting that most interspecific cladogenesis occurred well over 15 Ma.

Table 1 Pairwise comparisons of mitochondrial DNA sequences among Amazonian anoles. Uncorrected per cent sequence differences between haplotypes are given below the diagonal and maximum-likelihood corrected distances (HKY model) above the diagonal. Numbers following species names refer to map locations (Fig. 1)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1 *Anolis transversalis*	—	.30	.30	.33	.33	.30	.30	.30	.30	.72	.80	.77	.77	.70	.71	.74	.75	.76	.73	.74	.76	.75	.74	.75	.74	.72	.74	.74	.64	.64	.68	.68	.70	.84	.71	.70
2 *A. punctatus* 7	.18	—	.01	.09	.09	.05	.07	.07	.09	.70	.72	.77	.76	.74	.70	.68	.71	.72	.67	.67	.68	.72	.74	.74	.73	.72	.73	.74	.62	.62	.59	.59	.59	.77	.72	.72
3 *A. punctatus* 7	.17	.01	—	.09	.09	.05	.07	.07	.09	.72	.72	.77	.77	.74	.70	.68	.71	.71	.67	.67	.68	.71	.74	.74	.72	.72	.73	.74	.61	.61	.58	.58	.59	.77	.71	.71
4 *A. punctatus* 1	.19	.07	.07	—	.00	.09	.10	.10	.11	.75	.74	.79	.82	.76	.71	.69	.71	.71	.69	.68	.70	.73	.71	.75	.73	.73	.74	.72	.60	.60	.60	.60	.60	.77	.72	.72
5 *A. punctatus* 1	.19	.07	.07	.00	—	.09	.10	.10	.11	.75	.74	.79	.83	.76	.71	.69	.71	.71	.69	.68	.70	.72	.70	.74	.72	.73	.73	.71	.60	.60	.59	.59	.60	.77	.72	.72
6 *A. punctatus* 2	.17	.04	.04	.07	.07	—	.07	.07	.08	.76	.78	.81	.79	.77	.73	.71	.73	.74	.70	.70	.71	.73	.74	.74	.71	.72	.73	.74	.63	.63	.59	.59	.59	.82	.74	.74
7 *A. punctatus* 3	.17	.06	.06	.08	.08	.06	—	.00	.07	.68	.72	.73	.77	.70	.66	.65	.68	.68	.65	.65	.66	.68	.72	.70	.69	.68	.70	.71	.58	.58	.56	.56	.57	.75	.67	.67
8 *A. punctatus* 3	.17	.06	.06	.08	.08	.06	.00	—	.07	.68	.72	.74	.77	.70	.65	.65	.68	.68	.64	.65	.66	.68	.72	.70	.69	.68	.70	.71	.58	.58	.56	.56	.57	.74	.66	.67
9 *A. punctatus* 4	.17	.08	.08	.09	.09	.07	.06	.06	—	.71	.77	.75	.77	.71	.72	.70	.71	.71	.69	.69	.70	.72	.75	.75	.73	.74	.75	.74	.62	.62	.62	.62	.62	.77	.71	.70
10 *A. carpenteri*	.27	.26	.26	.27	.27	.27	.26	.26	.26	—	.37	.23	.22	.21	.27	.27	.27	.27	.28	.28	.28	.34	.32	.34	.35	.34	.35	.32	.45	.45	.48	.48	.47	.55	.49	.49
11 *A. humilis*	.28	.27	.27	.27	.27	.28	.27	.27	.28	.20	—	.37	.38	.36	.31	.32	.31	.31	.32	.31	.31	.39	.40	.41	.39	.39	.40	.40	.49	.49	.53	.53	.53	.58	.56	.56
12 *A. lemurinus*	.27	.27	.27	.28	.28	.28	.27	.27	.27	.15	.20	—	.23	.22	.29	.28	.29	.29	.30	.29	.28	.37	.37	.36	.35	.36	.35	.38	.48	.48	.55	.55	.55	.55	.53	.53
13 *A. limifrons*	.28	.27	.27	.28	.28	.27	.27	.27	.27	.15	.20	.15	—	.19	.27	.28	.28	.28	.28	.28	.28	.31	.32	.33	.33	.33	.33	.31	.46	.46	.51	.51	.49	.53	.50	.50
14 *A. trachyderma*	.26	.27	.27	.27	.27	.27	.26	.26	.26	.14	.19	.15	.14	—	.27	.27	.28	.28	.28	.28	.28	.30	.31	.31	.31	.31	.32	.31	.44	.44	.46	.46	.47	.49	.47	.48
15 *A. fuscoauratus* 2	.26	.26	.26	.26	.26	.27	.25	.25	.27	.17	.18	.17	.17	.17	—	.03	.04	.04	.04	.04	.03	.27	.30	.29	.29	.29	.30	.29	.44	.44	.45	.45	.46	.53	.45	.45
16 *A. fuscoauratus* 2	.27	.26	.26	.26	.26	.27	.25	.25	.26	.17	.18	.17	.17	.17	.03	—	.04	.04	.03	.03	.03	.28	.31	.30	.30	.29	.30	.28	.45	.45	.46	.46	.47	.49	.42	.42
17 *A. fuscoauratus* 1	.27	.27	.27	.27	.27	.27	.26	.26	.27	.17	.18	.17	.17	.17	.04	.04	—	.00	.04	.04	.04	.28	.30	.30	.29	.29	.30	.28	.46	.46	.46	.46	.46	.51	.43	.44
18 *A. fuscoauratus* 1	.27	.27	.27	.27	.27	.27	.26	.26	.27	.17	.18	.17	.17	.17	.04	.03	.00	—	.04	.04	.04	.28	.30	.30	.30	.30	.30	.29	.46	.46	.46	.46	.46	.51	.43	.44
19 *A. fuscoauratus* 3	.27	.26	.26	.26	.26	.26	.25	.25	.26	.17	.19	.18	.17	.17	.04	.03	.04	.04	—	.04	.03	.28	.31	.29	.29	.29	.29	.29	.46	.46	.46	.46	.46	.52	.44	.45
20 *A. fuscoauratus* 7	.27	.26	.26	.26	.26	.26	.25	.25	.26	.17	.18	.17	.17	.17	.04	.03	.04	.04	.04	—	.03	.28	.30	.29	.29	.28	.29	.28	.45	.45	.45	.45	.46	.52	.44	.44
21 *A. fuscoauratus* 4	.27	.26	.26	.27	.26	.26	.25	.25	.26	.17	.18	.17	.17	.17	.03	.03	.04	.04	.03	.03	—	.29	.31	.29	.29	.29	.30	.29	.46	.46	.45	.45	.46	.51	.44	.45
22 *A. ortonii* 1	.27	.26	.26	.27	.26	.26	.25	.25	.26	.19	.21	.20	.18	.18	.17	.17	.17	.17	.17	.17	.18	—	.09	.08	.08	.08	.08	.08	.45	.45	.47	.47	.48	.53	.49	.49
23 *A. ortonii* 3	.26	.27	.27	.26	.26	.26	.26	.26	.26	.18	.21	.20	.18	.18	.18	.18	.18	.18	.18	.17	.18	.08	—	.09	.09	.08	.09	.07	.46	.46	.47	.47	.48	.54	.51	.51
24 *A. ortonii* 6	.27	.26	.26	.27	.27	.26	.25	.25	.26	.19	.21	.20	.19	.18	.17	.18	.17	.17	.18	.18	.18	.07	.07	—	.01	.02	.02	.07	.46	.46	.46	.46	.48	.54	.49	.50
25 *A. ortonii* 7	.26	.26	.26	.26	.26	.26	.25	.25	.26	.19	.20	.19	.19	.18	.17	.18	.17	.17	.17	.17	.17	.07	.07	.01	—	.02	.01	.07	.46	.46	.46	.46	.48	.55	.50	.50
26 *A. ortonii* 7	.26	.26	.27	.26	.26	.25	.25	.25	.26	.19	.20	.20	.19	.18	.17	.17	.18	.18	.17	.17	.18	.07	.07	.02	.02	—	.02	.07	.44	.44	.46	.46	.47	.53	.48	.49
27 *A. ortonii* 7	.26	.26	.26	.27	.26	.26	.25	.25	.27	.19	.21	.19	.19	.18	.18	.18	.18	.18	.17	.17	.18	.07	.07	.02	.01	.02	—	.07	.47	.47	.47	.47	.49	.55	.50	.51
28 *A. ortonii* 2	.26	.27	.27	.26	.26	.26	.26	.26	.26	.18	.21	.20	.18	.18	.17	.17	.17	.17	.17	.17	.17	.07	.06	.06	.06	.06	.06	—	.48	.48	.48	.48	.49	.55	.49	.50
29 *A. nitens* 5	.25	.25	.25	.25	.25	.25	.24	.24	.25	.22	.23	.23	.22	.22	.22	.22	.23	.22	.23	.22	.23	.22	.22	.22	.22	.22	.23	.23	—	.00	.18	.18	.17	.40	.36	.36
30 *A. nitens* 5	.25	.25	.25	.25	.25	.25	.24	.24	.25	.22	.23	.23	.22	.22	.22	.23	.22	.23	.22	.22	.22	.22	.22	.22	.22	.22	.23	.23	.00	—	.18	.18	.17	.40	.36	.36
31 *A. nitens* 1	.26	.25	.24	.25	.25	.24	.24	.24	.25	.23	.24	.25	.23	.22	.22	.22	.22	.23	.22	.22	.22	.22	.22	.22	.22	.22	.22	.23	.13	.13	—	.00	.02	.43	.39	.39
32 *A. nitens* 1	.26	.25	.24	.25	.25	.24	.24	.24	.25	.23	.24	.25	.23	.22	.22	.22	.22	.23	.22	.22	.22	.22	.22	.22	.22	.22	.22	.23	.13	.13	.00	—	.02	.43	.39	.39
33 *A. nitens* 1	.26	.25	.24	.25	.25	.24	.24	.24	.25	.23	.24	.24	.23	.23	.23	.23	.23	.23	.23	.22	.23	.23	.23	.23	.23	.22	.23	.23	.12	.12	.02	.02	—	.42	.39	.40
34 *A. nitens* 2	.29	.28	.28	.28	.28	.29	.28	.27	.28	.25	.25	.25	.24	.23	.24	.23	.23	.23	.24	.24	.24	.24	.24	.24	.24	.24	.24	.24	.21	.21	.21	.21	.21	—	.17	.17
35 *A. nitens* 3	.27	.27	.27	.28	.27	.28	.26	.26	.28	.23	.25	.25	.24	.23	.22	.21	.22	.22	.22	.22	.22	.22	.22	.24	.23	.23	.23	.24	.20	.20	.21	.21	.21	.12	—	.00
36 *A. nitens* 3	.27	.27	.27	.28	.27	.28	.26	.26	.27	.23	.25	.25	.24	.23	.22	.21	.22	.22	.22	.22	.22	.24	.24	.23	.24	.23	.24	.23	.20	.20	.21	.21	.21	.13	.00	—

Discussion

High levels of mtDNA sequence divergence within and among Amazonian anole species strongly reject the hypothesis that these species separated initially during the Pleistocene. Divergences of mtDNA haplotypes among recognized species of Amazonian anoles exceed 15% in each case, indicating that their cladogenesis occurred in the Miocene or earlier. Haplotype differences among geographical populations within species range from 3 to 22%, indicating that even the most recent divergences sampled predate the Pleistocene, and in many cases the Pliocene. These results are concordant with molecular phylogenetic results from other rainforest vertebrates in rejecting the Pleistocene refuge hypothesis as an explanation for most geographical variation and speciation (Moritz *et al.* 2000).

Furthermore, our results suggest that evolutionary differentiation among geographical populations within the four widely codistributed Amazonian anoles has been temporally disjunct; these species differ in per cent sequence difference among haplotypes sampled from the same geographical locations. This temporal discordance precludes a common explanation for geographical diversification in these species, indicating separate times of arrival in Amazonia and lineage-specific responses to historical geographical barriers.

Amazonian anoles clearly do not form a monophyletic group (Fig. 2), indicating repeated faunal exchanges with Central America and Caribbean islands. Although the large genetic divergences reported strongly reject the timescale presented by Vanzolini & Williams (1970), the relative sequence of events they suggested, a long history of *Anolis punctatus* in Amazonia and successive migration of *A. nitens*, *A. ortonii* and finally *A. fuscoauratus* into Amazonia, is compatible with our results.

The ancestral lineage of *A. punctatus* may have the longest history in South America. *A. punctatus* is the sister taxon to the endemic South American species, *A. transversalis*, suggesting a South American distribution for their most recent common ancestor over 15 Ma (Table 1). Geographical genetic diversity within *A. punctatus* suggests that this species is at least 6 Myr old, and perhaps much older.

Vanzolini & Williams (1970) proposed that the remaining Amazonian anoles (*A. fuscoauratus*, *A. nitens*, *A. ortonii* and *A. trachyderma*) are part of a group that arose in Mexico/Central America and entered South America in separate migrations. Our phylogeny is compatible with this geographical scenario, but does not exclude the alternative hypothesis of a South American origin for this group followed by migration to Central America.

As suggested by Vanzolini & Williams (1970), *A. nitens* appears older than *A. fuscoauratus* and *A. ortonii*, and may have originated over 15 Ma (Table 1). Extreme genetic differentiation among geographical populations of *A. nitens* suggests that its geographical morphological diversification also probably preceded the Pleistocene. Our results cannot reject hypotheses that older refugia, associated with pre-Pleistocene climatic cycles, initiated evolutionary divergence among these populations, or that Pleistocene refugia permitted persistence of these ancient lineages (Haffer 1997). Low genetic diversity within *A. fuscoauratus* supports the hypothesis that this species is a relatively recent invader of South America from Central America (Vanzolini & Williams 1970). Relatively low geographical differentiation among haplotypes within *A. fuscoauratus* is compatible with the finding that it is the most continuously distributed of the geographically widespread Amazonian anoles (Avila-Pires 1995) and also generally the most abundant anole throughout its range.

High levels of genetic and morphological divergence observed among geographical populations of *A. nitens* question its systematic status. Morphological diversity in *A. nitens* has been used to diagnose five subspecies (Vanzolini & Williams 1970; Avila-Pires 1995), which are also ecologically differentiated (Vitt & Zani 1996; Vitt *et al.* 2001). Three recognized subspecies are represented in this study (*A. nitens nitens*, *A. n. scypheus*, *A. n. tandai*). The Acre and Amazonas populations form a strongly supported monophyletic group traditionally recognized as *A. n. tandai*. The Roraima population represents *A. n. nitens*, and the Ecuadorian population represents *A. n. scypheus*. Further study of geographical genetic interactions among these subspecies probably will reveal that they are distinct species. Current taxonomy therefore probably underestimates species diversity for Amazonian anoles.

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Appendix I

Museum numbers and localities for tissue from which DNA was extracted for this study (LSU = Louisiana State University Museum of Natural Science Collection of Genetic Resources; KdQ = Kevin de Queiroz, field series) and GenBank accession numbers (in parentheses):

A. Geographic sampling of widespread Amazonian species

1. Ecuador, Sucumbios Province, Reserva Faunistica Cuyabeno: *Anolis fuscoauratus* LSU H-12538 (AF337789), LSU H-12545 (AF337788); *A. nitens* LSU H-12543 (AF337802), LSU H-12592 (AF337804), LSU H-12650 (AF337803); *A. ortonii* LSU H-12747 (AF337793); *A. punctatus* LSU H-12751 (AF337772), LSU H-12577 (AF337773).

2. Brazil, Acre State, ≈ 5 km N Porto Walter, inland from the Rio Juruá: *A. fuscoauratus* LSU H-13566 (AF337786), LSU H-13801 (AF337787); *A. nitens* LSU H-13850 (AF337805); *A. ortonii* LSU H-13904 (AF337799); *A. punctatus* LSU H-13910 (AF337774).

3. Brazil, Amazonas State, Rio Ituxí at the Madeirera Scheffer: *A. fuscoauratus* LSU H-14094 (AF337790); *A. nitens* (1) LSU H-14096 (AF337807) (2) LSU H-14097 (AF337806); *A. ortonii* LSU H-14099 (AF337794); *A. punctatus* (1) LSU H-14101 (AF337775) (2) LSU H-14102 (AF337776).

4. Brazil, Rondônia State, Parque Estadual Guajara Mirim, ≈ 90 km N Nova Mamore: *A. fuscoauratus* LSU H-15471 (AF337792).

5. Brazil, Roraima State, Fazenda Nova Esperanca: *A. nitens* LSU H-12298 (AF337800), LSU H-12378 (AF337801).

6. Brazil, Pará State, Alter de Chão: *A. ortonii* LSU H-14163 (AF337795).

7. Brazil, Pará State, Agropecuaria Treviso: *A. fuscoauratus* LSU H-14327 (AF337791); *A. nitens* LSU H-13850 (AF337805); *A. ortonii* LSU H-14313 (AF337798), LSU H-14406 (AF337797), LSU H-14444 (AF337796); *A. punctatus* LSU H-14336 (AF337770), LSU H-14453 (AF337771).

B. Outgroup taxa (newly reported sequences)

Phenacosaurus nicefori, Colombia, J. Renjifo 2537 (AF337768); *A. mestrei*, Cuba, KdQ 1709 (AF337779); *A. sagrei*, Cuba, KdQ 1797 (AF337778); *A. carpenteri*, Nicaragua, LSU H-14688 (AF337781); *A. lemurinus* (AF337782); *A. lineatus*, Curacao, H-5450 Shochat (AF337784) (Shochat & Dessauer 1981); *A. trachyderma*, Brazil, Pará State, LSU H-14300 (AF337785); *A. woodi*, Costa Rica, M. Butler 2/21/95 (AF337780), *A. transversalis*, Brazil, Acre State, LSU H-13893 (AF337769).

C. Outgroup taxa from previous studies (Jackman et al. 1999, 2001)

Polychrus acutirostris AF055925, *A. aeneus* AF055950, *A. agassizi* AF055952, *A. grahami* AF294299, *A. lineatopus* AF294295, *A. humilis* AF055944, *A. microtus* AF055947, *A. occultus* AF055977.