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Out of Cuba: overwater dispersal and speciation among lizards in the *Anolis carolinensis* subgroup

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Abstract

Overwater dispersal and subsequent allopatric speciation contribute importantly to the species diversity of West Indian *Anolis* lizards and many other island radiations. Here we use molecular phylogenetic analyses to assess the contribution of overwater dispersal to diversification of the *Anolis carolinensis* subgroup, a clade comprising nine canopy-dwelling species distributed across the northern Caribbean. Although this clade includes some of the most successful dispersers and colonists in the anole radiation, the taxonomic status and origin of many endemic populations have been ambiguous. New mitochondrial and nuclear DNA sequences from four species occurring on small islands or island banks (*Anolis brunneus*, *Anolis longiceps*, *Anolis maynardi*, *Anolis smaragdinus*) and one species from the continental United States (*A. carolinensis*) are presented and analysed with homologous sequences sampled from related species on Cuba (*Anolis allisoni* and *Anolis porcatus*). Our analyses confirm that all five non-Cuban species included in our study represent distinct, independently evolving lineages that warrant continued species recognition. Moreover, our results support Ernest Williams's hypothesis that all of these species originated by overseas colonization from Cuban source populations. However, contrary to Williams's hypothesis of Pleistocene dispersal, most colonization events leading to speciation apparently occurred earlier, in the late Miocene–Pliocene. These patterns suggest that overwater dispersal among geologically distinct islands and island banks is relatively infrequent in anoles and has contributed to allopatric speciation. Finally, our results suggest that large Greater Antillean islands serve as centres of origin for regional species diversity.

Keywords: *Anolis*, biogeography, dispersal, speciation, West Indies

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Introduction

Allopatric speciation following overwater colonization often contributes importantly to the species diversity of islands and island archipelagos (Givnish *et al*. 1995; Hollocher 1998; Steppan *et al*. 2003; Gillespie 2004). Recent attempts to integrate such speciation into island biogeographical theory suggest that speciation is unlikely when dispersal is frequent (due to the homogenizing effects of recurrent gene flow) but becomes more common as the rate of dispersal declines (Whittaker 1998; Heaney 2000; Lomolino 2000). Among West Indian *Anolis* lizards, which have long played an important role in the development of island biogeographical theory (e.g. Darlingon 1957; MacArthur & Wilson 1967), high levels of island endemicity suggest sporadic overwater dispersal followed by divergence in allopatry (Williams 1969; Pregill & Crother 1999). However, a recent molecular analysis of brown anoles (*Anolis sagrei*) on the Great Bahama bank suggests that overwater dispersal is more common than previously expected and plays an underappreciated role in constraining species-level divergence among populations on different islands (Calsbeek & Smith 2003).

Here we use phylogenetic analyses of mitochondrial (mtDNA) and nuclear (nDNA) sequence data to test the contribution of overwater dispersal to species diversification in the green canopy-dwelling anoles of the *carolinensis* subgroup (Burnell & Hedges 1990). Members of this clade are considered among the best overwater dispersers and colonists in the anole radiation and occur naturally on Cuba, numerous smaller islands in the northern Caribbean, and

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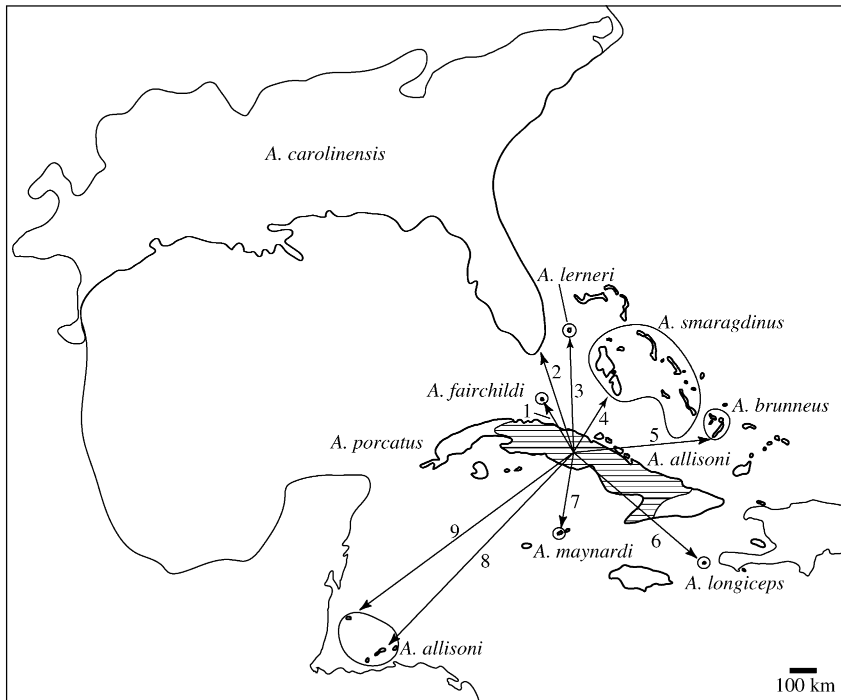

Fig. 1 Distribution of the *carolinensis* subgroup. Arrows represent dispersal events hypothesized by Williams (1969, 1989). Cuba is the only island where species in this subgroup occur sympatrically: *Anolis porcatus* is distributed islandwide, with the exception of a narrow gap in eastern Cuba, whereas *Anolis allisoni* is restricted to central Cuba (indicated by horizontal bars). *Anolis lerneri* was later demoted to a subspecies of *Anolis smaragdinus* (Williams 1976). Distribution of *Anolis carolinensis* in North America follows Conant & Collins (1991).

the southeastern United States (Fig. 1). *Anolis carolinensis* is particularly noteworthy because it is the only species of anole native to the continental United States where it occurs in the Pleistocene fossil record (Holman 1995). Because most of the areas occupied by the *carolinensis* subgroup have never been connected by dry land, overwater dispersal *per se* is uncontroversial (Williams 1969, 1989). However, the contribution of such dispersal to the *carolinensis* subgroup's species diversity and the geographical and temporal patterns of colonization events are poorly understood. Ernest Williams (1969, 1989) used morphological similarities to suggest up to nine independent overwater colonization events from a Cuban centre of origin during the Pleistocene, seven of which led to speciation events (Fig. 1). We test three specific predictions of this 'out-of-Cuba' hypothesis.

First, this hypothesis predicts that overwater dispersal followed by allopatric speciation produced seven non-Cuban species, all of which are allopatrically distributed and endemic to a single island or island bank. However, the taxonomic status of these populations has always been tentative because they are allopatrically distributed and often difficult to distinguish morphologically (Williams 1969; Schwartz & Thomas 1975). Indeed, one species recognized by Williams in 1969 (*Anolis lerneri*) has since been demoted to a subspecies of *Anolis smaragdinus* (Schwartz & Thomas 1975; Williams 1976), and additional species recognized by Williams and others (e.g. Schwartz & Henderson 1991; Powell *et al*. 1996) are considered subspecies of a polytypic *A. carolinensis* by other authors (Chan *et al*. 1987; Schoener & Adler 1991; Schoener 1994; Losos & de Queiroz 1997; Spiller *et al*. 1998). Meanwhile, populations from islands off the coast of Central America, which are currently considered conspecific with Cuban *Anolis allisoni*, may warrant status as a distinct species (Ruibal & Williams 1961). We present the first detailed analysis of species boundaries in the *carolinensis* subgroup by sampling mtDNA and nDNA within and among populations and testing which populations represent independently evolving evolutionary lineages that warrant recognition under the general lineage concept of species (de Queiroz 1998, 1999).

The second prediction of the out-of-Cuba hypothesis is that the *carolinensis* subgroup's distribution represents multiple colonization events from a Cuban centre of origin. This scenario predicts that non-Cuban species are phylogenetically more closely related to Cuban populations than they are to one another. We test this direct, independent-colonization scenario against three alternatives, which feature one or more instances of secondary dispersal or stepping-stone colonization. Because phylogenetic tests of such colonization scenarios require comprehensive sampling of potential source populations (Emerson 2002), we sample DNA sequences from non-Cuban populations that are homologous to ones used in a previous study of Cuban populations of the *carolinensis* subgroup (Glor *et al*. 2004).

Finally, we test the third prediction that overwater dispersal in the *carolinensis* subgroup occurred during the Pleistocene (Williams 1969). This prediction is based primarily on the assumption that the areas occupied by the *carolinensis* subgroup, with the exceptions of Cuba and the

United States, were intermittently submerged prior to the Pleistocene (Williams 1969). However, recent geological and biogeographical evidence suggests longer continuous emergence for some of these islands. For example, portions of the Cayman Islands have likely been continually emergent since the Pliocene (Askew 1994; Hess *et al*. 1994; Proctor 1994) and Navassa may have been continually emergent for as long as 5 million years (Myr) (Powell 1999). Moreover, a molecular-clock-based analysis of allozymic data suggests that divergence between Cuban populations and *A. carolinensis* in the United States predates the Pleistocene and occurred perhaps sometime in the Pliocene (Buth *et al*. 1980).

Methods

Specimens examined

Outgroup taxa include all three members of the *isolepis* subgroup (*Anolis isolepis*, *Anolis oporinus*, and *Anolis altitudinalis*), which is the sister clade to the *carolinensis* subgroup, and members of the more inclusive *carolinensis* series (*Anolis alutaceus* and *Anolis loysiana*) (Burnell & Hedges 1990). Ingroup sampling includes 174 individuals sampled from across Cuba for a previous study (Glor *et al*. 2004), 30 individuals representing five of the six non-Cuban species (samples of *Anolis fairchildi* from Cay Sal were unavailable), and the Central American island population of the otherwise Cuban *Anolis allisoni* (Fig. 2). Our sampling includes several representatives of each species and typically multiple individuals from each population sampled (Fig. 2). Specimens examined are listed below with associated voucher information (USNM, United States National Museum, Washington, D.C.; JBL, Jonathan B. Losos field series; JJK, Jason J. Kolbe field series) and GenBank Accession numbers for rhodopsin and mtDNA, respectively: three *Anolis maynardi* from Little Cayman Island (JBL 400–402; AY902438-AY902439; AY902409–AY902411); two *Anolis longiceps* from Navassa Island (USNM 549614–549615; AY902436–AY902437; AY902407–AY902408); five *Anolis brunneus*, three from Crooked Island (USNM 548475–549476, 549478; AY902440–AY902441; AY902412–AY902414) and two from Acklins Island (USNM 549506, 549508; AY902442–AY902443; AY902415–AY902416); two *Anolis smaragdinus smaragdinus* from Staniel Cay (USNM 549512–549513; AY902445; AY902423–AY902424); six *A. smaragdinus lerneri*, two from South Bimini (USNM 549536–549537; AY902446–AY902447; AY902417–AY902418), two from Andros Island [USNM 549569–549570; AY902419–AY902420 (mtDNA only)], and two from Chub Cay (USNM 549558–549559; AY902444; AY902421–AY902422); 10 *Anolis carolinensis* from the United

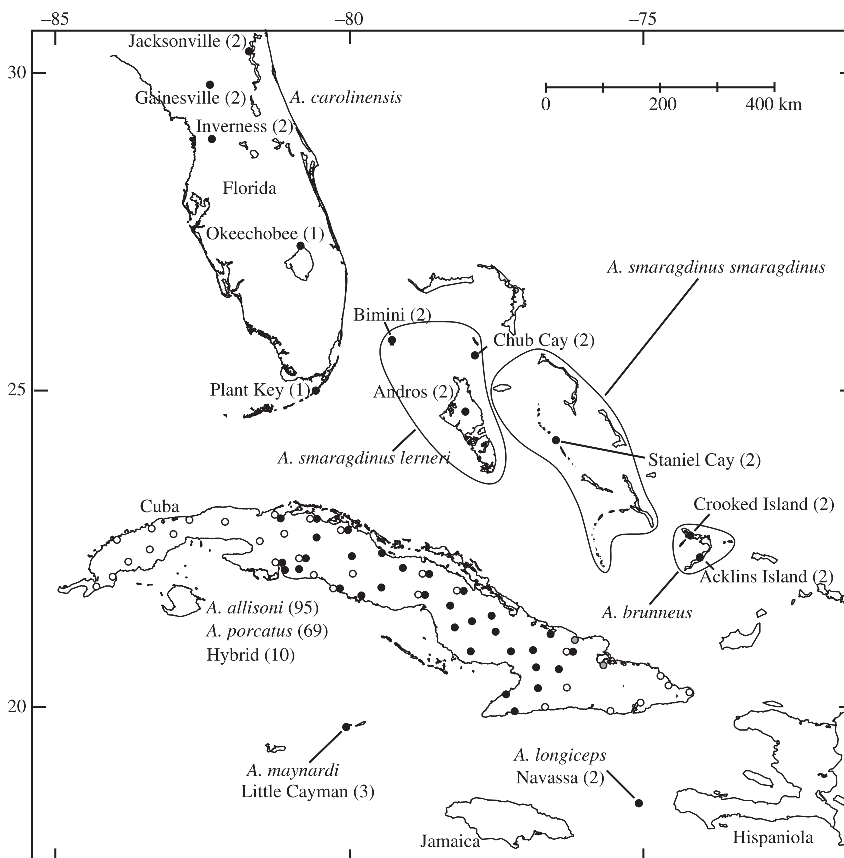

Fig. 2 Sampling for this study. Circles indicate sampling localities. On Cuba filled circles represent *Anolis allisoni*, open circles *Anolis porcatus*, and grey circles hybrids of these two species. Numbers in parentheses indicate sample sizes. Samples from the Central American population of *A. allisoni* and Louisiana *Anolis carolinensis* are not included on this map.

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States, three from Louisiana [JBL 671, 982, 984; AY902425–AY902427 (mtDNA only)] and seven from Florida, one from Plant Key (JJK 395; AY902451; AY902428), one from Okeechobee (JJK 480; AY902452; AY902429), two from Inverness (JJK 566–567; AY902453; AY902430–AY902431), two from Gainesville (JJK 587–588; AY902449–AY902450; AY902432–AY902433), and one from Jacksonville (JJK 634; AY902448; AY902434). Blood samples from two live *A. allisoni* collected in 1996 from Isla Guanaja (one of the Bay Islands of Honduras) were obtained from a private collection [AY902435 (mtDNA only)].

Molecular methods

Genomic DNA was obtained from liver or tail tissue using the Viogene DNA Extraction Miniprep System (Viogene). Polymerase chain reaction (PCR) amplification began with 180 s at 95 °C followed by 30 cycles of 95 °C for 35 s, 52–60 °C for 35 s, and 72 °C for 150 s. Reactions with volumes of 25 or 50 µL included 1–2 µL of genomic DNA and a mixture of 49.5% H_2O, 10% M190G thermophilic DNA polymerase 10× buffer, 25 mm $MgCl_2$, dNTPs, and 2 pmol primer mix, and 0.5% Promega *Taq* DNA polymerase (Promega Corporation). We cleaned amplified products using the Viogene Gel-M™ Gel Extraction System (Viogene). We ran sequencing reactions using BigDye Terminator Ready Reaction Kits (PerkinElmer) and an MJ Research BaseStation automated sequencer (MJ Research).

mtDNA

We amplified a *c*. 1200-bp fragment of mtDNA using the PCR protocol above with an annealing temperature of 52 °C, a reaction volume of 50 µL, and two previously published primers: L4437 (Macey *et al*. 1997) and H5730 (Glor *et al*. 2004). These primers and one additional primer, either L4882a (Macey *et al*. 2000) or L4882c (Glor *et al*. 2004), were used to sequence the entire 1200-bp fragment, which includes complete sequence for the genes encoding ND2 and $tRNA^{Trp}$ and partial sequence for the $tRNA^{Ala}$ gene. Sequences were aligned by eye using structural models for tRNA genes (Kumazawa & Nishida 1993; Macey *et al*. 1997).

Rhodopsin gene

Primers in the third and fourth exons of the nuclear-encoded rhodopsin gene (*Rod3* and *Rod4*; Glor *et al*. 2004) were used to amplify a *c*. 1000-bp fragment of this gene consisting of the third intron and portions of flanking exon sequence. Twenty-five-microlitre PCRs were run with an annealing temperature of 60 °C. Resulting bands were cored from 2.5% low-melt agarose gels and reamplified in 50 µL reactions. Sequencing was conducted using the same two primers used for amplification.

Phylogenetic analyses

We used a conditional-combination approach to determine whether mtDNA and rhodopsin-gene data sets should be combined for phylogenetic analyses (reviewed in Huelsenbeck *et al*. 1996). Concordance is assessed using the incongruence length difference (ILD) test (Farris *et al*. 1994) as well as both Templeton (1983) and SH (Shimodaira & Hasegawa 1999) tests (*sensu* Larson 1994). These tests were conducted in PAUP* 4.0b10 (Swofford 2002) (ILD test = partition homogeneity test in PAUP*). Initially we conducted these analyses on all 66 taxa common to both data sets. We then repeated these tests after removing Cuban individuals containing introgressed mtDNA haplotypes due to hybridization between *A. allisoni* and *A. porcatus* (Glor *et al*. 2004).

Phylogenetic trees were constructed using both maximum-parsimony and Bayesian criteria for each data set. Redundant sequences were excluded from all analyses. We conducted parsimony analyses in PAUP* using tree-bisection–reconnection (TBR) branch-swapping and 10 random-addition replicates; because numerous shallowly divergent taxa produce lengthy parsimony searches (without improvement of the overall tree score), all parsimony analyses were limited to 1×10^8 rearrangements. Two hundred bootstrap replicates (Felsenstein 1985) with five random additions per replicate and decay indices ('branch support' of Bremer 1994) assessed support for individual nodes. MACCLADE 4 (Maddison & Maddison 2000) generated constraint trees for the decay-index analyses.

MRMODELTEST 1.1B (http://www.ebc.uu.se/systzoo/staff/nylander.html), a modified version of MODELTEST (Posada & Crandall 1998), conducted hierarchical hypothesis testing of alternative models of evolution to determine the appropriate model of evolution for Bayesian analysis. MRBAYES 3.0 (Huelsenbeck & Ronquist 2001) generated Bayesian trees by running four chains for 10 000 000 generations and sampling every 10 000 generations. Burn-in points were determined by plotting parameters from each sampled tree in TRACER version 1.1 (http://evolve.zoo.ox.ac.uk/software.html?id = tracer) and visualizing trees in the TREE SET VIZ 2.0 module (http://comet.lehman.cuny.edu/treeviz/software.html) of MESQUITE (Maddison & Maddison 2003). Posterior-probability values indicate support for all nodes in the Bayesian topology.

Hypothesis testing

Templeton (1983) and SH (Shimodaira & Hasegawa 1999) tests were used to assess species monophyly and to discriminate specific colonization scenarios under parsimony and likelihood-based criteria, respectively. Because likelihood searches for alternative topologies were not possible due to the size of the data sets, SH tests were conducted by comparing the optimal tree generated in MRBAYES to alternative topologies estimated using maximum parsimony.

We tested the hypothesis that each non-Cuban population results from an independent colonization by a Cuban source population (Fig. 1) against three alternative hypotheses that invoke secondary dispersal among non-Cuban portions of the range. The direct, independent-dispersal scenario makes the explicit phylogenetic prediction that non-Cuban species will be more closely related to Cuban populations than they are to one another. Our first alternative hypothesis posits that the United States was colonized via the Bahamas rather than directly from Cuba and predicts that *A. carolinensis* will be more closely related to one or more Bahamian species than to Cuban populations. Our second alternative asks whether two Bahamian species (*A. brunneus* and *A. smaragdinus*) result from independent Cuban dispersal events. These populations occupy two geologically distinct island banks (Great Bahama and Acklins) that have likely never been connected by dry land but are arranged north of Cuba along an east–west axis that may facilitate dispersal via prevailing westerly currents (Hedges 1996; Calsbeek & Smith 2003). If secondary dispersal occurred among banks, the Bahamian species should be more closely related to one another than to Cuban species. Our third alternative hypothesis predicts that morphologically similar taxa occupying small islands south of Cuba (*A. maynardi* on Little Cayman and *A. longiceps* on Navassa) represent a single dispersal from Cuba and subsequent dispersal between these smaller islands. Dispersal among these small, remote island populations is suggested by their shared characteristics of extraordinarily long snouts, leading some to propose that *A. longiceps* and *A. maynardi* are sister species (Schmidt 1921; Thomas 1966; Powell 1999).

Results

Incongruence of data

Phylogenetic congruence between the mtDNA data and the rhodopsin-gene data is consistently rejected. First, concordance across all 66 taxa common to both data sets is rejected under the ILD test ($P < 0.001$) as well as Templeton and SH tests ($P < 0.0001$ in each case). This incongruity persists even when 12 introgressed mtDNA haplotypes are excluded (ILD: $P = 0.001$; Templeton and SH tests: $P < 0.0001$ in each case). We therefore conduct phylogenetic analyses on each data set independently and discuss concordance and discordance on a case-by-case basis.

mtDNA

Our aligned mtDNA data set of 1173 sites comprises 179 previously published and 30 new sequences. All new sequences are unique with the following two exceptions: all three *Anolis maynardi* sequences are identical as are both sequences from *Anolis smaragdinus lerneri* on Bimini and both *Anolis allisoni* from Isla Guanaja. Absence of premature stop codons, a strong bias against guanine in the light strand, and functional stability of the tRNA genes suggest that these sequences are authentic mitochondrial DNA (Zhang & Hewitt 1996).

MRMODELTEST selects the GTR + I + Γ model for Bayesian analyses. These analyses produce a well-resolved strict-consensus tree with a mean ln-likelihood score of –14975.672 (SD = 2.319) and mean parameter values of alpha = 0.404, proportion invariant = 0.280, G ↔ T = 1.000, C ↔ T = 10.961, C ↔ G = 0.902, A ↔ T = 0.983, A ↔ G = 34.273, A ↔ C = 1.076 following a burn-in period of 200 000 generations (Fig. 3). The effective sample size of the 979 post burn-in trees is 100.548. Parsimony analysis of 499 parsimony-informative characters produces > 10 000 equally most parsimonious trees of 2632 steps. Because Bayesian and maximum-parsimony phylogenies are highly congruent, only the Bayesian tree is presented, with support from the parsimony analyses indicated where the two trees are concordant (Fig. 3).

Rhodopsin gene

Our aligned rhodopsin-gene data set comprises 82 previously published and 18 new sequences. Twenty-nine redundant sequences are excluded from all analyses, resulting in an aligned data set of 71 unique sequences 1764-bp long. The species *Anolis longiceps*, *Anolis maynardi*, and *Anolis brunneus* each contain a single unique rhodopsin-gene sequence for the specimens sampled. *Anolis smaragdinus* contains two unique sequences, one found on Bimini and one on Chub and Staniel Cay. The two rhopopsin-gene sequences sampled from the Gainesville population of *Anolis carolinensis* are identical, and the Jacksonville *A. carolinensis* rhodopsin-gene sequence is identical to a sequence on GenBank lacking locality data (Kawamura & Yokoyama 1994). MRMODELTEST selects the HKY + I + Γ model for Bayesian analyses. MRBAYES produces a well-resolved strict consensus tree with a mean ln-likelihood score of –3890, following a burn-in period of 40 000 generations (Fig. 4). The effective sample size of the 995 post burn-in trees estimated in TRACER is 618.805. Model parameters for the likelihood model are alpha = 0.201, proportion invariant = 0.726, kappa = 4.369. Analysis of 106 parsimony-informative characters produces > 10 000 equally most parsimonious trees of 258 steps. As with mtDNA, Bayesian and parsimony analyses are highly congruent; only the Bayesian tree is presented here, with support from parsimony analyses shown where the two trees are concordant (Fig. 4).

Phylogenetic relationships in the carolinensis *subgroup*

Although phylogenetic congruence between the mtDNA and rhodopsin-gene (nDNA) data is rejected, these markers

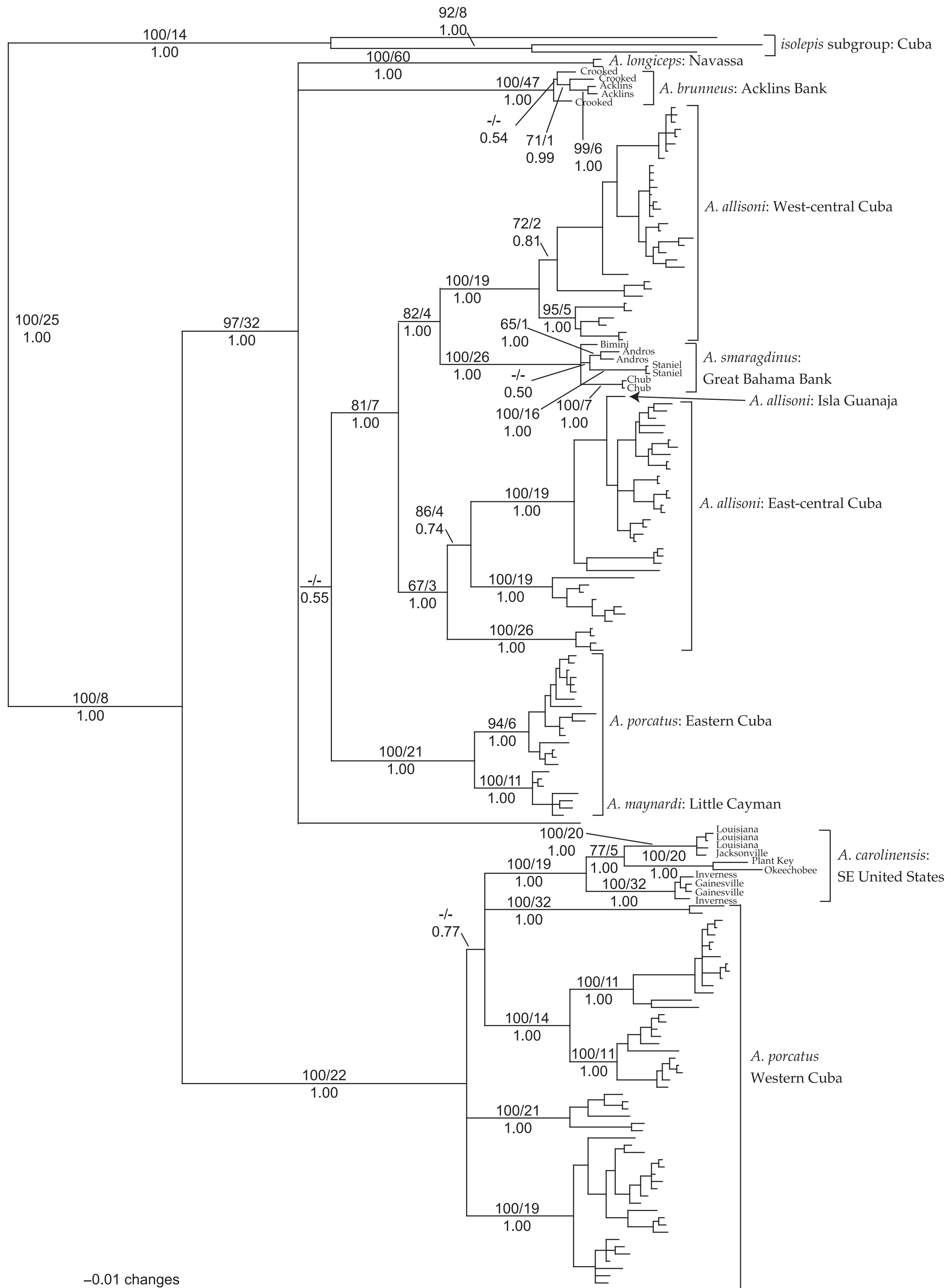

Fig. 3 Mitochondrial DNA phylogram inferred using Bayesian analysis with posterior probabilities noted below branches whose posterior probabilities exceed 0.5. Nonparametric bootstrap (Felsenstein 1985) and Bremer support (Bremer 1994) are marked above branches whose bootstrap values exceed 0.5 in a parsimony analysis. Outgroup species belonging to the more inclusive *carolinensis* series (*Anolis alutaceus* and *Anolis loysiana*) are not shown.

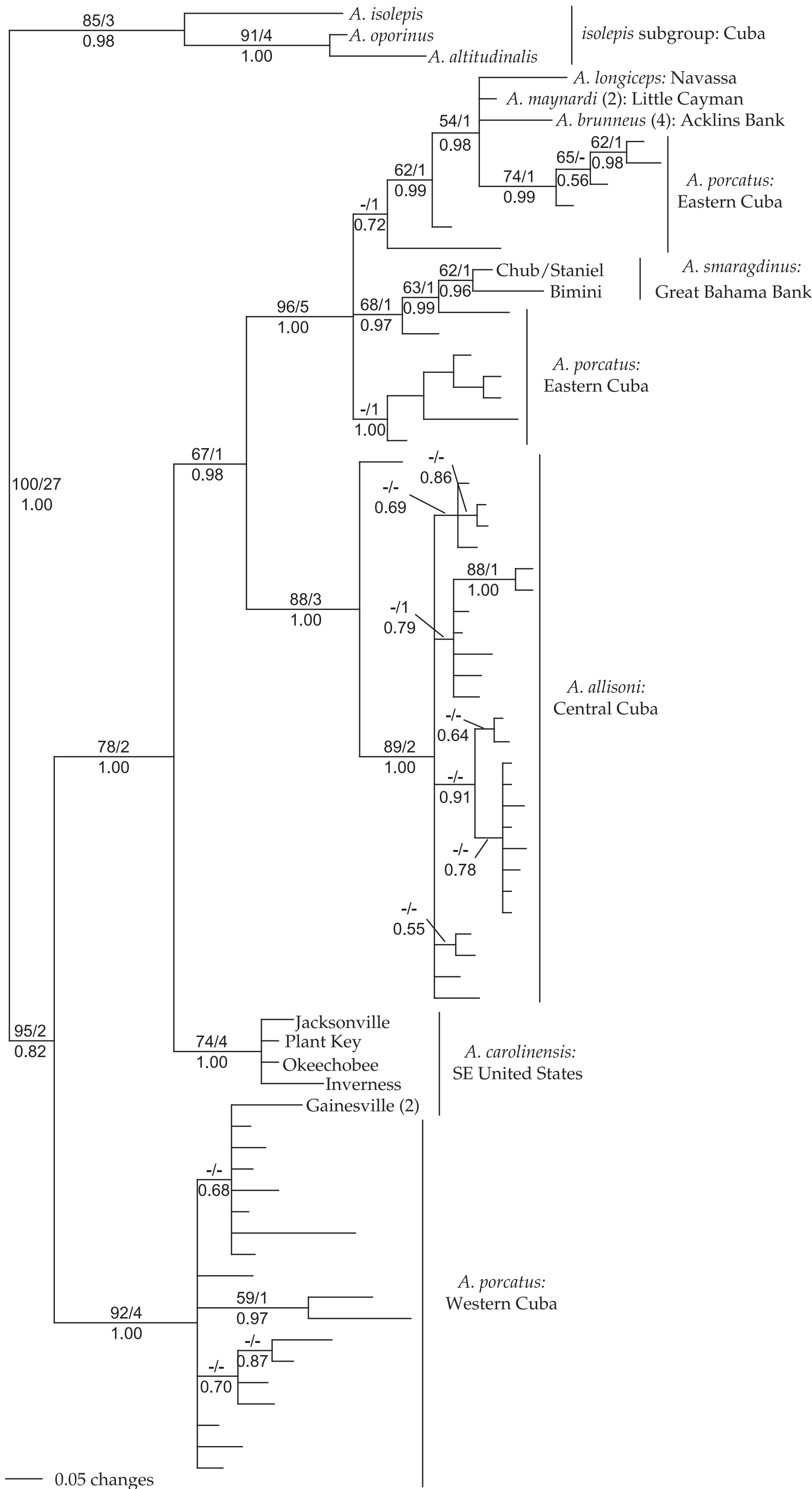

Fig. 4 Rhodopsin-gene phylogram inferred using Bayesian analysis with posterior probabilities noted below branches whose posterior probabilities exceed 0.5. Nonparametric bootstrap support (Felsenstein 1985) and Bremer support (Bremer 1994) are marked above branches whose bootstrap values exceed 0.5 in a parsimony analysis. Outgroup species belonging to the more inclusive *carolinensis* series (*Anolis alutaceus* and *Anolis loysiana*) are not shown.

recover many common topological features (Figs 3 and 4). First, both markers recover monophyletic *isolepis* and *carolinensis* subgroups, which are strongly supported as sister taxa (Figs 3 and 4). Moreover, relationships among the three species in the *isolepis* subgroup are concordant across markers (Figs 3 and 4). Among Cuban members of the *carolinensis* subgroup (*Anolis allisoni* and *Anolis porcatus*), both markers recover the same well-supported and deeply

Table 1 Mean pairwise divergence for mtDNA. Tamura-Nei corrected distances are below diagonal, uncorrected distances above. Bold values in diagonal represent Tamura-Nei corrected intragroup divergences. 'Outgroups' category refers to two members of the more inclusive *carolinensis* series (*Anolis alutaceus* and *Anolis loysiana*)

		1	2	3	4	5	6	7	8	9	10	11	12
1	Outgroups	**0.255**	0.211	0.217	0.201	0.201	0.197	0.205	0.21	0.202	0.2	0.207	0.197
2	*isolepis* subgroup	0.256	**0.128**	0.173	0.168	0.169	0.155	0.171	0.167	0.161	0.164	0.167	0.163
3	*A. longiceps*	0.265	0.206	**0.004**	0.111	0.12	0.109	0.151	0.114	0.144	0.113	0.113	0.105
4	*A. maynardi*	0.242	0.197	0.125	—	0.106	0.1	0.145	0.104	0.137	0.109	0.103	0.102
5	*A. brunneus*	0.241	0.196	0.137	0.119	**0.015**	0.108	0.145	0.113	0.14	0.12	0.112	0.101
6	*A. smaragdinus*	0.237	0.178	0.123	0.11	0.121	**0.019**	0.132	0.084	0.124	0.08	0.082	0.089
7	*A. carolinensis*	0.247	0.201	0.175	0.168	0.167	0.15	**0.048**	0.138	0.087	0.136	0.136	0.132
8	*A. allisoni* (Isla Guanaja)	0.256	0.197	0.129	0.117	0.129	0.092	0.161	—	0.129	0.089	0.038	0.086
9	Western *A. porcatus*	0.242	0.187	0.166	0.157	0.16	0.141	0.096	0.149	**0.07**	0.126	0.131	0.127
10	Western *A. allisoni*	0.241	0.19	0.129	0.122	0.136	0.087	0.156	0.099	0.143	**0.038**	0.089	0.089
11	Eastern *A. allisoni*	0.251	0.197	0.129	0.116	0.128	0.09	0.159	0.041	0.151	0.1	**0.053**	0.089
12	Eastern *A. porcatus*	0.236	0.19	0.119	0.114	0.113	0.099	0.152	0.097	0.145	0.1	0.1	**0.040**

Table 2 Mean pairwise divergence for the rhodopsin-gene sequences. Tamura-Nei corrected distances are below the diagonal, and uncorrected distances above. Bold values in the diagonal represent Tamura-Nei corrected intragroup divergences. 'Outgroups' category refers to two members of the more inclusive *carolinensis* series (*Anolis alutaceus* and *Anolis loysiana*)

		1	2	3	4	5	6	7	8	9	10
1	Outgroups	**0.055**	0.1	0.109	0.105	0.106	0.113	0.103	0.098	0.108	0.112
2	*isolepis* subgroup	0.107	**0.015**	0.035	0.03	0.035	0.039	0.031	0.03	0.035	0.036
3	*A. longiceps*	0.119	0.036	—	0.005	0.008	0.016	0.025	0.032	0.011	0.027
4	*A. maynardi*	0.113	0.031	0.005	—	0.004	0.01	0.019	0.027	0.006	0.021
5	*A. brunneus*	0.115	0.036	0.008	0.004	—	0.012	0.024	0.031	0.01	0.025
6	*A. smaragdinus*	0.123	0.04	0.016	0.01	0.013	**0.004**	0.022	0.033	0.01	0.024
7	*A. carolinensis*	0.112	0.032	0.025	0.019	0.024	0.022	**0.008**	0.018	0.021	0.02
8	Western *A. porcatus*	0.106	0.03	0.033	0.028	0.032	0.034	0.018	**0.006**	0.03	0.03
9	Eastern *A. porcatus*	0.117	0.036	0.011	0.006	0.01	0.01	0.021	0.031	**0.008**	0.022
10	*A. allisoni*	0.122	0.037	0.027	0.021	0.025	0.024	0.02	0.03	0.023	**0.005**

divergent clades identified from the same data in a previous analysis (Glor *et al*. 2004). Mitochondrial DNA identifies four distinct clades, which, despite some discordance due to mtDNA introgression, closely correspond to eastern and western populations of both *A. porcatus* and *A. allisoni* (Glor *et al*. 2004) (Fig. 3). The nDNA data concordantly diagnose three of these clades, representing *A. allisoni* and eastern and western populations of *A. porcatus* (Glor *et al*. 2004) (Fig. 4). The allopatrically distributed eastern and western populations of *A. porcatus* are distinct with respect to mtDNA, nDNA, and morphology and likely warrant status as distinct species (Glor *et al*. 2004). Samples from the Isla Guanaja population of *A. allisoni*, from which only mtDNA sequences are obtained, are deeply nested within, and only shallowly divergent from, sequences sampled from eastern Cuban populations of *A. allisoni*.

Each non-Cuban species for which multiple haplotypes are found is recovered as a well-supported monophyletic group of haplotypes by both markers (Table 3, Figs 3 and 4), with the exception of nDNA sequences from *A. carolinensis* (see below). Moreover, samples from each non-Cuban species are deeply divergent from one another and all Cuban samples (Tables 1 and 2).

Mitochondrial DNA and nuclear DNA sequences sampled from Cuba form a paraphyletic group with respect to all six non-Cuban species (Figs 3 and 4). Mitochondrial DNA haplotypes from North American *A. carolinensis* form a clade nested with strong support among haplotypes from the western population of *A. porcatus* (Fig. 3, Table 3). However, the pattern observed among nDNA sequences sampled from this species is more complex (Fig. 4). Nuclear DNA sequences obtained from the Gainesville population are placed in a position similar to the mtDNA haplotypes, but other *A. carolinensis* nDNA sequences are placed outside the western *A. porcatus* clade as the sister group to a clade containing the remaining ingroup taxa (Fig. 4).

Table 3 Results of hypothesis testing using the methods of Templeton (1983) and Shimodaira & Hasegawa (1999). A significant result denotes rejection of the altenative hypothesis as stated in favour of the results shown in Figs 3 and 4

	mtDNA			Rhodopsin gene		
	Templeton		SH	Templeton		SH
Alternative hypothesis	*N**	*P*	*P*	*N**	*P*	*P*
Species monophyly†						
1. Nonmonophyly of *A. brunneus*‡	46	**< 0.0001**	**< 0.003**	—	—	—
2. Nonmonophyly of *A. carolinensis*	15	< 0.0522	**< 0.039**	6	< 0.1573	< 0.318
3. Nonmonophyly of *A. longiceps*‡	59	**< 0.0001**	**< 0.001**	—	—	—
4. Nonmonophyly of *A. smaragdinus*	22	**< 0.0007**	**< 0.035**	2	0.4795	< 0.934
Direct colonization						
5. Nonmonophyly of *A. carolinensis* + western Cuban *A. porcatus*	22	**< 0.0015**	< 0.077	2	0.5930	< 0.648
6. Nonmonophyly of *A. smaragdinus* + Cuban populations§	4	0.3711	< 0.755	2	0.4795	< 0.958
Stepping stone/ secondary dispersal						
7. Monophyly of *A. carolinensis* + one or both Bahamian species	54–71	**< 0.0001**	**< 0.001**	17–18	**< 0.0005**	**< 0.002**
8. Monophyly of Bahamian species (*A. brunneus* and *A. smaragdinus*)	19	< 0.0519	< 0.245	5	< 0.1655	< 0.641
10. Monophyly of populations from islands south of Cuba (*A. longiceps* and *A. maynardi*)	7	< 0.4295	< 0.920	0¶	—	—

*Difference in lengths of most parsimonious and alternative topologies.
†Single unique mtDNA and rhodopsin-gene sequences were obtained from *A. maynardi*, which is not included in this table.
‡Only one unique rhodopsin sequence.
§Test differed between mtDNA and rhodopsin. mtDNA haplotypes from *A. smaragdinus* are nested within a group of haplotypes from *A. allisoni*, whereas rhodopsin-gene sequences from *A. smaragdinus* are nested within a group containing sequences from *A. porcatus*.
¶Arrangements with *A. longiceps* and *A. maynardi* as sister taxa are equally parsimonious to arrangements where they are not sister taxa.

These remaining ingroup taxa (*A. longiceps*, *A. maynardi*, *A. brunneus*, *A. smaragdinus*, *A. allisoni* and eastern *A. porcatus*) form a clade in both the mtDNA and nDNA analyses (Figs 3 and 4). In the mtDNA tree, this large clade contains a basal polytomy of the lineages ancestral to *A. longiceps*, *A. brunneus*, *A. maynardi*, eastern *A. porcatus*, and *A. smaragdinus*/ *A. allisoni* (Fig. 3). Meanwhile, nDNA identifies a poorly resolved basal divergence separating *A. allisoni* from the remaining ingroup taxa (Fig. 4). The phylogenetic position of sequences from the four small-island endemics is either poorly resolved by both markers (*A. brunneus*, *A. longiceps*, *A. maynardi*) or conflicts between markers (*A. smaragdinus*) (Figs 3 and 4). Neither marker recovers any well-supported monophyletic groups of species containing exclusively non-Cuban taxa (Figs 3 and 4, Table 3).

For both markers, sequences from *A. smaragdinus* are most closely related to Cuban sequences, but the markers conflict regarding which Cuban sequences (Figs 3 and 4). Mitochondrial DNA recovers *A. smaragdinus* as the sister taxon to a group of haplotypes from western populations of *A. allisoni* with moderate support (Fig. 3). Nuclear DNA suggests, with weak support, that *A. smaragdinus* sequences are most closely related to two sequences sampled from the eastern population of *A. porcatus* (Fig. 4).

Although mtDNA also recovers monophyletic groups of haplotypes for each island from which *A. smaragdinus* is sampled, the two subspecies representing this species (*A.* s. *smaragdinus* and *A. s. lerneri*) do not form reciprocally monophyletic groups of haplotypes and are only slightly divergent (Table 1, Fig. 3). All three alternatives to direct dispersal are rejected. Both markers reject the hypothesis that the two Bahamian endemics (*A. smaragdinus* and *A. brunneus*) form a monophyletic group (Table 3). Moreover, both markers reject a close relationship between *A. carolinensis* and either or both Bahamian taxa (Table 3). Relationships among *A. longiceps*, *A. maynardi*, *A. brunneus* and closely associated samples are unresolved by both markers. The two long-snouted forms from islands south of Cuba do not form a monophyletic group, but neither marker can reject this hypothesis statistically (Table 3).

Discussion

Species diversity

All five non-Cuban members of the *carolinensis* subgroup included in this study are deeply divergent with respect to both mtDNA and nDNA (Tables 1 and 2). In the case of mtDNA, uncorrected sequence divergences exceeding 8% distinguish each species; such levels of mitochondrial divergence are typical among congeneric reptile species (Johns & Avise 1998) and indicate pre-Pleistocene evolutionary separation (see following discussion).

Although our intraspecific sampling is limited, four of the five non-Cuban species are monophyletic with respect to both mitochondrial and nuclear DNA haplotypes (Table 3, Figs 3 and 4). The lone exception to this pattern, *Anolis carolinensis*, is not monophyletic with respect to nDNA (Fig. 4). However, two lines of evidence suggest that this pattern could result from incomplete lineage sorting, which is expected to result in polyphyletic or paraphyletic relationships among haplotypes sampled from diverging populations prior to the achievement of reciprocal monophyly (Neigel & Avise 1986). First, such lineage sorting is expected to take longer for nuclear than mitochondrial markers (Moore 1995), which could account for the monophyly of mitochondrial, but not nuclear, sequences sampled from *A. carolinensis*. Second, ancestral polymorphisms are expected to persist longer in populations characterized by larger effective sizes (Hare *et al*. 2002), providing an explanation for the nonmonophyly of a species that is widespread and common throughout the southeastern United States (*A. carolinensis*), but not species endemic to small islands or island banks.

Overall, the deep genetic divergences among, and monophyly within, the currently recognized species combines with previously documented morphological (Schwartz & Henderson 1991) and allozymic (Webster *et al*. 1972; Buth *et al*. 1980) differentiation to support continued species-level recognition of these populations. This result suggests that dispersal among geologically distinct islands or island banks is infrequent and has not prevented the divergence of numerous isolated populations.

Nevertheless, our results do leave open the possibility that dispersal is more common among islands located within the same bank. Although our sampling is limited, both mitochondrial and nuclear sequences are poorly differentiated across the ranges of both *Anolis smaragdinus* and *Anolis brunneus* on the Great Bahama and Acklins Banks, respectively (Table 1, Fig. 4). In *A. smaragdinus*, but not *A. brunneus*, mtDNA haplotypes sampled within islands form weakly differentiated monophyletic groups (< 3% uncorrected divergence) (Table 2, Fig. 3). These patterns suggest either ongoing overwater dispersal among islands sharing the same bank, as suggested by Calsbeek & Smith (2003) for brown anoles (*Anolis sagrei*) from the Great Bahama Bank, or historical admixture when the banks formed single landmasses during Pleistocene sea-level fluctuations. Further intraspecific sampling is required to clarify dispersal by members of the *carolinensis* series among islands on the same bank.

Pattern of dispersal

Sequences sampled from a source population that has provided colonists for multiple small islands often form a paraphyletic group with respect to sequences sampled from the colonized areas (e.g. Melnick *et al*. 1993; Thorpe *et al*. 1994). In the *carolinensis* subgroup, mitochondrial and nuclear DNA haplotypes sampled from Cuba are paraphylyetic with respect to all five non-Cuban populations. This pattern, combined with the fact that most close relatives of the *carolinensis* subgroup are Cuban, strongly supports the hypothesis of a Cuban centre of origin. Moreover, our phylogenetic analyses reject all three hypotheses involving secondary dispersal or stepping-stone colonization and suggest a minimum of four independent dispersals from Cuba.

Three non-Cuban populations are most closely related to Cuban populations and are likely the result of direct dispersal. One such dispersal led to the mainland species, *A. carolinensis*. All mtDNA haplotypes and two of the six rhodopsin-gene sequences obtained from this species are nested with strong support within samples from the western Cuban population of *A. porcatus*, suggesting direct dispersal from western Cuba to the continental United States (Table 3, Figs 3 and 4). In addition, our analyses specifically reject the hypothesis that the United States was colonized in a stepping-stone fashion via the Great Bahama or Acklins Banks (Table 3). Colonization of the United States via Cay Sal, which is home to *Anolis fairchildi* (the only species in the *carolinensis* subgroup unavailable for our study), is unlikely because *A. fairchildi* is morphologically more similar to *A. smaragdinus* from the Great Bahama Bank than to either Cuban or US populations (Schwartz & Thomas 1975).

Although colonization of mainland areas by island taxa is considered less likely than colonization in the opposite direction (Brown & Lomolino 1998), successful West Indies to mainland America colonization is not unique to *A. carolinensis*. Indeed, a number of other plants and animals have colonized Florida via the West Indies (e.g. Reiskind 2001; Santiago-Valentin & Olmstead 2004). In addition, another independent colonization of the mainland by a West Indian anole permitted an evolutionary radiation of anoles throughout Central and South America (Nicholson *et al*. 2005).

A second direct dispersal from Cuba produced colonization of the Great Bahama Bank and subsequent evolution of *A. smaragdinus*. Both mtDNA and nDNA sequences sampled from this species form a clade nested within Cuban populations (Figs 3 and 4). Although the two markers disagree over which Cuban populations are the source (Figs 3 and 4), our analyses suggest that colonization of the Great Bahama Bank via another small island or island bank is unlikely. Colonization via the Acklins Bank, in particular, is not supported (Table 3). Sequences sampled from the two geologically distinct Bahamian banks do not form a monophyletic group based on either mtDNA or rhodopsin-gene sequences (Figs 3 and 4), and grouping them requires 19 additional steps in parsimony analyses of mtDNA, resulting in a marginally nonsignificant P value from the Templeton test (Table 3).

A third independent dispersal directly from Cuba occurred to Isla Guanaja, which is located off the coast of

Honduras. This event appears to have occurred relatively recently because the single mtDNA haplotype obtained from both of the Isla Guanaja *A. allisoni* sampled is deeply nested within, and relatively shallowly divergent from, eastern Cuban populations of this species (Fig. 3, Table 1). Our analyses cannot discriminate the hypothesis that Central American *Anolis allisoni* populations represent natural dispersal and deserve species-level recognition (Ruibal & Williams 1961) from the alternative hypothesis that these populations represent human-mediated dispersal.

At least one additional dispersal from Cuba is required to establish species endemic to Navassa, Little Cayman, and the Acklins Bank (*A. longiceps*, *A. maynardi*, *A. brunneus*). However, the source population for these three species is unresolved by analyses of both mtDNA and nDNA (Figs 3 and 4). As a result, we cannot assess whether these species represent several independent dispersals directly from Cuba or secondary dispersals among smaller islands. We cannot reject the hypothesis that the long-snouted species on remote islands south of Cuba (*A. longiceps* and *A. maynardi*) are sister taxa resulting from dispersal among these islands following a single colonization directly from Cuba (Table 3).

Both of these species and *A. brunneus* from the Acklins Bank form part of a large polytomy that also includes Cuban populations and characterizes both data sets. Such polytomies result from either an inability of the data to resolve sequential divergence events (e.g. a soft polytomy) or multiple contemporaneous divergence events (e.g. a hard polytomy). Three lines of evidence outlined by previous authors (Maddison 1989; Jackman *et al*. 1999; Slowinski 2001; Poe & Chubb 2004) suggest that the polytomy among sequences sampled from *A. brunneus*, *A. longiceps*, *A. maynardi* and certain Cuban populations form a hard polytomy. First, both molecular markers independently recover polytomies including similar taxa (Figs 3 and 4). Second, the branches dividing the polytomy are extremely short in individual Bayesian and parsimony reconstructions, and the zero-branch-length test (Poe & Chubb 2004; see also Slowinski 2001) suggests that the lengths of these branches are not significantly different from zero. Finally, nodes older and younger than the polytomy are recovered with strong support, suggesting that the molecular markers employed are capable of resolving dichotomous branching events at this scale.

Contemporaneous divergence of three small-island endemics indicates that a single historical event may underlie the origins of all three species. Because Navassa, Little Cayman, the Acklins Bank, and Cuba have likely never been connected by dry land, any such historical event would have required overwater dispersal. In the case of Navassa and Little Cayman, colonization by Cuban populations likely required dispersal against prevailing currents, which generally sweep across Cuba heading northwest (Hedges 2001). Hurricane-mediated dispersal is one possible explanation for such colonization events (Powell 1999; Hedges 2001).

This polytomy, however, is not necessarily an indication that the rate of overwater colonization has changed over time. Another possibility is that early colonization events established *carolinensis*-group species on the different land areas, and that these established populations then prevented later colonists from establishing new populations in the same areas. Phylogeographical studies of anoles on Cuba and Hispaniola (Glor *et al*. 2003; Kolbe *et al*. 2004) and patterns of anole species diversity (e.g. Williams 1969; Losos *et al*. 1993) suggest that established populations often prevent close relatives from expanding into their territories. If adjacent island territories are colonized within a short period of evolutionary time and anole species established, later colonists are unlikely to become established even if rate of overseas dispersal remains constant through time.

Timing of divergence events

The mean uncorrected mitochondrial genomic divergence between each non-Cuban member of the *carolinensis* subgroup and its closest Cuban relative ranges from 8.7% to 10.9% (Table 1). Based on a molecular-clock calibration of 1.3% pairwise divergence per Myr, derived from a similar mitochondrial gene region in other reptiles and amphibians (Macey *et al*. 1998; Weisrock *et al*. 2001), these levels of divergence suggest that each species has been separated from its closest relatives for more than 6 Myr. Our results therefore place species diversification in the *carolinensis* subgroup prior to the Pleistocene. Prior suggestions that the Pleistocene was an important period of diversification for this clade are based largely on the assumption that the low-lying areas occupied by this clade were submerged as recently as 20 000 BP (Williams 1969; Webster *et al*. 1972). Our molecular-clock-based analysis supports conclusions of other recent studies that some of the islands occupied by the *carolinensis* subgroup have been emergent much longer. For example, portions of the Cayman Islands (Askew 1994; Hess *et al*. 1994; Proctor 1994) and Navassa (Powell 1999) are now thought to have been continuously emergent since the Pliocene. Further intraspecific sampling and additional genetic markers may clarify the temporal sequence of dispersal events by members of the *carolinensis* subgroup. Although timing of specific dispersal events may be overestimated when haplotypes are not sampled comprehensively, our estimates are concordant with earlier molecular studies (Buth *et al*. 1980) in rejecting a late-Pleistocene origin for species-level divergences in the *carolinensis* subgroup.

Conclusions

Our results reinforce the importance of overwater dispersal to species diversification in *Anolis* lizards. Specifically, our

analyses support two of the three predictions made by the out-of-Cuba hypothesis for diversification of the *carolinensis* subgroup of anoles. First, overwater dispersal and subsequent divergence in geographical isolation have produced five species in this clade that are endemic to small islands, island banks, or the continental United States. Second, all of the non-Cuban members of the *carolinensis* subgroup are derived from Cuban source populations. However, the third aspect of the out-of-Cuba hypothesis, the proposed Pleistocene timing of these events, is rejected by our analyses. Our results suggest instead that most species diversification in this group occurred in the late Miocene-Pliocene. Overall, our results suggest that overwater dispersal by *Anolis* lizards is relatively infrequent among geologically distinct islands or island banks and may lead to allopatric speciation. Moreover, our results suggest that large Greater Antillean islands are important centres of origin for regional species diversity. In addition to the species in the *carolinensis* subgroup, eight additional anole species endemic to small islands in the northern Caribbean also have close affinities to Greater Antillean taxa and are likely the result of similar processes (Losos & de Queiroz 1997).

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