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The Phylogenetic Relationships Of Native And Introduced *Anolis* Lizards On Cay Sal Bank, Bahamas And On Grand Cayman, Cayman Islands

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Abstract

Anolis lizards in the West Indies are one of the best-studied examples of adaptive radiation. Speciation and diversification in the Caribbean islands have led to the present recognition of hundreds of anole species. Anoles have evolved independently on many of these islands, particularly on the Greater Antillean islands, including Cuba. The Cuban anoles evolved *in situ*, yet have since spread to other regions, including the Bahamas, the Cayman Islands, and the Cay Sal Bank. The Cay Sal Bank encompasses a small ring of islands located between Florida, USA and Cuba, and is politically part of the Bahamas. Within these originally Cuban *Anolis* lineages, two species occur on Cay Sal Bank: the brown anole, *Anolis sagrei,* and the green anole, *Anolis fairchildi*. It is unknown whether these two anole species arrived to Cay Sal Bank from Cuba to the south or the Bahamas to the east. Additionally, it is not clear whether *Anolis fairchildi*, a Cay Sal endemic, is a valid species. On the island of Grand Cayman, an unidentified species of green anole has recently been introduced. The same question applies as to where this species originated and to what species it belongs. To examine all of these alternatives, genetic data was generated for the mitochondrial locus *ND2* from *A. fairchildi*, *A. sagrei*, three unknown samples from Grand Cayman, and related green and brown anole species. The resulting sequences were aligned with data from other West Indian anoles and a series of phylogenetic trees was constructed to examine these relationships. The divergence times were estimated for these species using calibrated molecular clock analyses in the program BEAST. The data suggest that both *A. fairchildi* and *A. sagrei* colonized Cay Sal Bank from western Cuba, not the Bahamas, during the mid-to-late Pleistocene. Based on phylogenetic relationships of the Cuban anoles, *A. fairchildi* is not a valid species, and has recently diverged and formed a small clade within the larger Cuban *A. porcatus* group. Using forensic phylogenetics, the three Grand Cayman green anole sequences identified as Cuban *A. porcatus*. One of these sequences is sister to the western Cuba *A. porcatus* and the other two are sister to the introduced Dominican Republic *A. porcatus*, clouding the likely origins of the population. Future work might include additional sampling of introduced *A. porcatus*.

1. Introduction

Caribbean *Anolis* lizards, a genus of iguanian lizards belonging to the Dactyloidae family, are excellent models of adaptive radiation[1]. The genus *Anolis* contains over 300 species in the Caribbean, and on Cuba alone there are more than 60 species[2,3]. As these are abundant, these species have been heavily studied in the context of ecology and evolution[1]. Among these islands, replication of morphology and ecology are seen, but although these species are similar, they are not all phylogenetically related. Due to their prolific diaspora and limited DNA sampling, some groups among both brown and green anoles have ambiguous phylogenetic relationships.

Over millions of years, anoles have proliferated throughout the Caribbean region, resulting in species colonizing Cuba, the Bahamas, Central America, the Cayman Islands, and the southeastern United States due to two mechanisms: sympatric and allopatric speciation. Through sympatric speciation, different populations of the same species become adapted to different features of their environment resulting in speciation, while with allopatric speciation, a physical barrier separates two populations[4]. Physical separation and overwater dispersal contribute to the majority of diversification of West Indian *Anolis* lizards[1]. In this study, I investigate the origins of three populations of anoles, which have never been studied using genetic data. I focus on two species from a remote island archipelago, the Cay Sal Bank, located between Cuba and the Florida Peninsula. I also focus on an introduced population on the island of Grand Cayman.

1.1 Origin of Green Anoles

On the island of Cuba, examples exist of both overwater dispersal and physical separation leading to the production of eight recognized species of green anoles (Fig. 1). Members of these species complexes are distributed not only on Cuba, but also the Bahamas, the southeastern United States, the Cayman Islands, and coastal Central America[5,6]. While the native Cayman Island green anole, *Anolis maynardi*, inhabits the island of Little Cayman, human mediated dispersal has resulted in an unknown green anole species being introduced in the last ten years to the island of Grand Cayman (Figure 2A). To protect the native anole species on the island (*Anolis conspersus*), the Cayman Islands Department of Environment needs to determine the introduced species identity and where it originated to prevent further introduction. The first objective of this study is to determine the origin and identity of the introduced Grand Cayman green anoles.

The second objective focuses on a small population of green anoles, *A. fairchildi*, that inhabits Cay Sal Island (Fig. 2B). Little is known about this species of anole other than they are large in size relative to other green anoles and morphologically similar to the Bahamian green anole, *Anolis smaragdinus*. The timing of their colonization is unknown, as well as whether this species is more closely related to the green anoles of Cuba or the Bahamas. The validity of the species is also under question, given these uncertainties. Due to patterns of dispersal among the Cuban green anoles, it is likely that *A. fairchildi* arrived on Cay Sal Bank through direct dispersal from western Cuba. Alternatively, they could have arrived on the bank from the Bahamas involving secondary dispersal. From Cuba, *A. smaragdinus* colonized the Bahamas. Colonization of Cay Sal Bank via the United States is unlikely due to the ocean currents and the morphology of *A. fairchildi*.

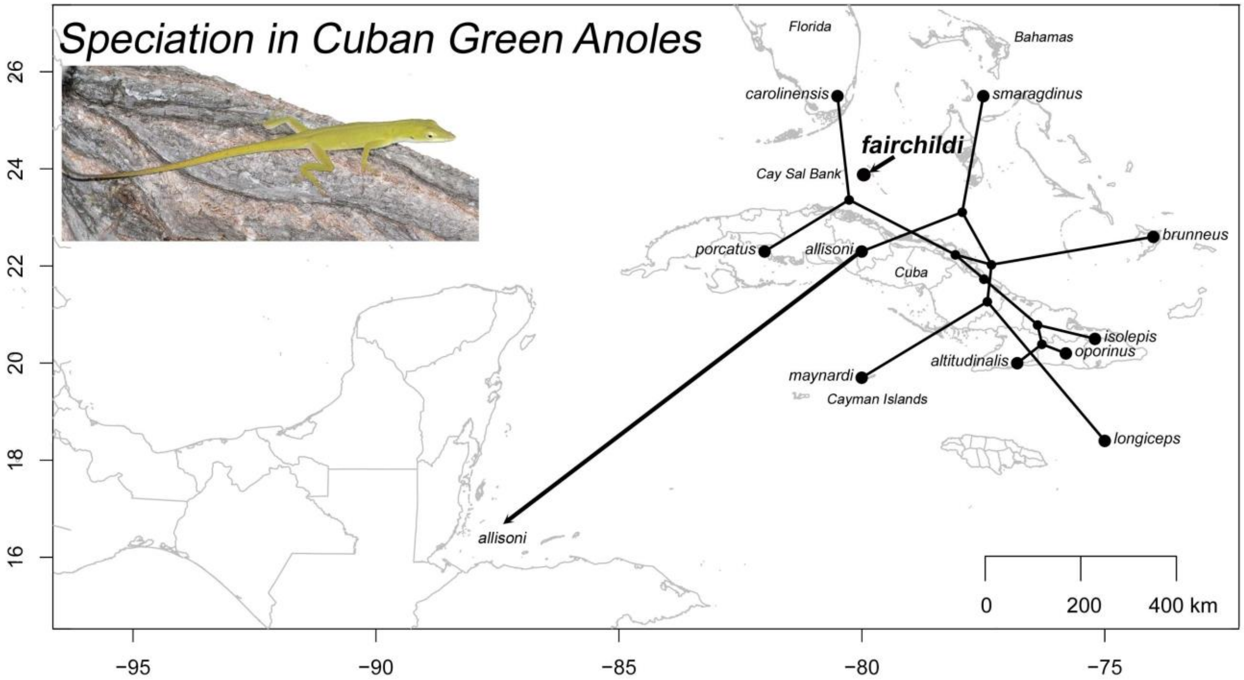

Figure 1. Evolutionary (phylogenetic) relationships among the 8 recognized Cuban green anoles (and close relatives). Here the species are represented as labelled dots on the map, while interconnecting lines represent the relationships among the species, with inferred ancestors as unlabelled dots. Note the location of the Cay Sal Bank, the Cayman Islands, and that *Anolis fairchildi* on the Cay Sal Bank has unknown evolutionary affinities. (Map after R.G. Reynolds and based on data from [6]).

1.2 Origin of Brown Anoles

The third objective is to determine the origin of the Cuban brown anole, *Anolis sagrei*, population that inhabits Cay Sal Bank (Fig. 2C). The diversification of *A. sagrei* has similar dispersal patterns to the Cuban green anole, leading to a wide dispersion of relatively ancient and recent lineages across the northern Caribbean and substantial variation in genetic divergence and morphology. Interestingly, in some populations dispersal through hurricanes has been suggested to mediate the diversification of this species by influencing the gene flow[7]. *Anolis sagrei* has been studied phylogenetically, and the species most likely evolved on Cuba and scattered among the northern Caribbean islands through the process of overwater dispersal[8]. However, even though *A. sagrei* is capable of dispersing widely, they appear to have diverged into west and east populations across the island of Cuba[8,9]. The two populations originated in the early Pliocene on Cuba then colonized northern islands of the Caribbean. Although *A. sagrei* is divergent on the island of Cuba and across the Greater Antillean Islands, some established populations are able to interbreed. Therefore, there is continued debate concerning the number of species within the *A. sagrei* clade. Some populations are deeply divergent and qualify as a valid species under the phylogenetic species concept, but not under the biological species concept.

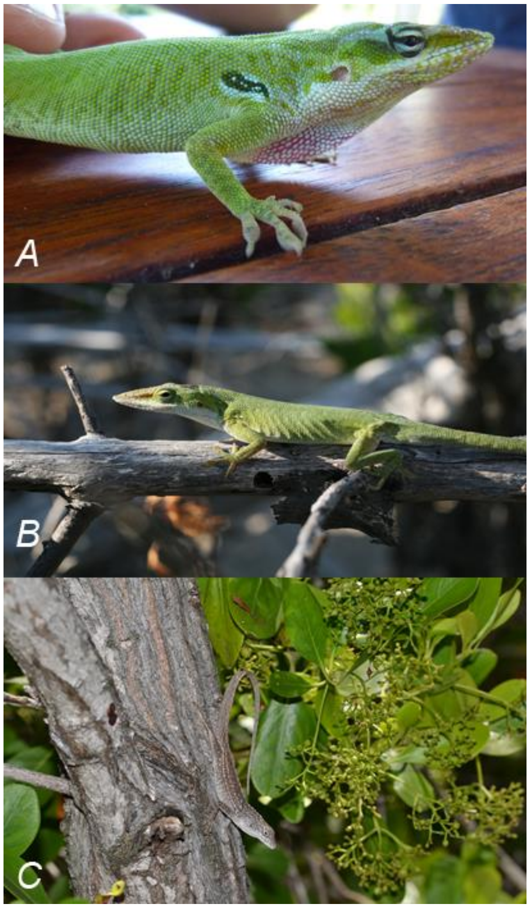

Figure 2. A) Introduced Grand Cayman anole (photo from www.anoleannals.org), B) Cay Sal Bank Green Anole *Anolis fairchildi* (Photo by R.G. Reynolds), C) Cay Sal Bank Brown Anole *Anolis sagrei* (Photo by A.R. Puente-Rolon).

1.3 Geologic and Geographic Description of Cay Sal

Cay Sal Bank, a 6000km ^{2} detached carbonate platform, forms a small ring of islands located between Florida, USA and Cuba (Fig. 3). The bank is politically part of the Commonwealth of the Bahamas and rarely visited by scientists due to dangerous illegal activities occurring there. At present the bank has few exposed islands due to its geological history and topography. These islands suggest the early stages of bank drowning, which occurs when sea levels rise faster than the carbonate production and accumulation[10]. Therefore, these tiny islands are slowly becoming submerged. Studies using satellite-derived DEM found that during the Holocene, around 11,000 years BP, only 1% of the platform was inundated. Conversely, 6,000 years BP approximately 99% of platform was inundated. Cay Sal Bank has deep-water margins of 15 m to 30 m and lacks platform-edge reefs, unlike the neighbouring Great and Little Bahamas banks[11]. The largest island, Cay Sal Island, is located on the southwestern edge of the bank and is vegetated with silver palms, mangroves, dense shrubs and grasses providing an ideal habitat for both *A. sagrei* and *A. fairchildi*.

The currents around the bank provide valuable insight for predicting patterns of overwater dispersal. Three major currents, the Florida Current, Nicholas Channel, and the Santaren Channel surround Cay Sal Bank. The Florida Current, a branch of the Gulf Stream, flows northeastward to northward with a rate of flow of 0.7-1.0 m/sec[11]. The Nicholas Channel flows eastward toward the Great Bahamas Bank with a rate of flow of 0.1- 0.3 m/sec, and the Santaren Channel flows north and then loops back toward Cay Sal Bank at 0- 0.1 m/sec[11]. The flow direction and rate of these currents would suggest overwater dispersal patterns from Cuba to Cay Sal via the Nicholas channel, despite the present classification of anoles from Cay Sal as being of Bahamian origin via the Santaren Channel. Alternatively, current flow via the Santaren Channel is slow and could easily carry propagules from the western Great Bahamas Bank to Cay Sal Bank.

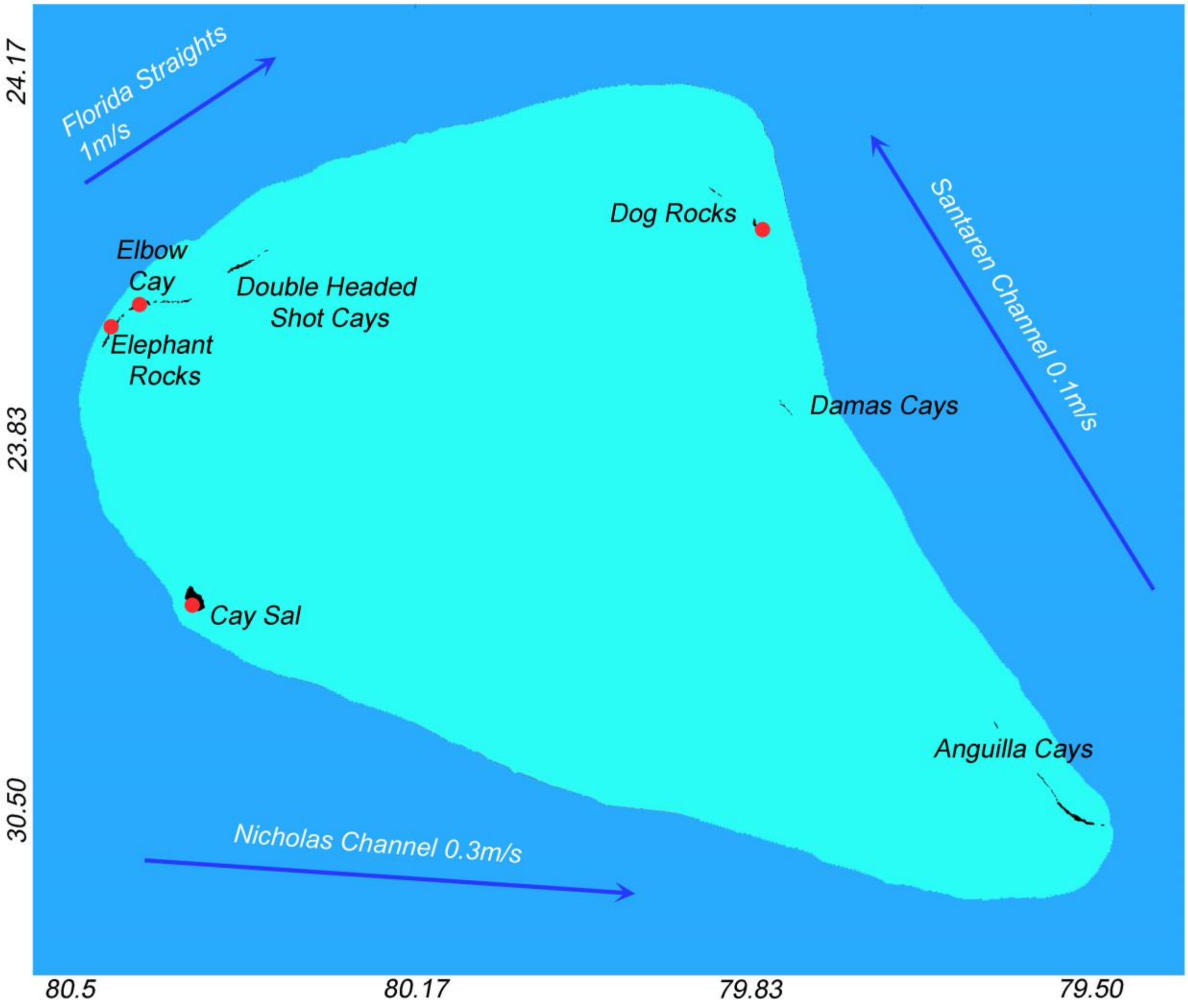

Figure 3. Locations of islands on the Cay Sal Bank, with red dots representing islands where anoles were collected in 2015. Current vectors of channels surrounding Cay Sal Bank are also shown.

Using phylogenetic analyses of the mitochondrial locus ND2, I hypothesized that phylogenetic relationships of Cay Sal green and brown anoles would reflect the current standing taxonomy. As it currently stands, *Anolis fairchildi* is thought to represent a distinct species derived from Bahamian ancestors, while Cay Sal brown anoles represent members of the Bahamian brown anole subspecies *A. sagrei ordinatus*, as they are currently recognized. Finally, I hypothesized that I would be able to ascertain the origins of the Grand Cayman anoles using forensic genetic techniques combined with a comprehensive molecular alignment for the *A. carolinensis* green anole clade.

2. Methods

2.1 Field Work

I travelled to the Cayman Islands in May 2016 with my mentor, Dr. Reynolds, and a team of herpetologists led by Dr. Anthony Geneva from Harvard University. On Grand Cayman, I obtained three non-native green anoles from scientific officers at the Cayman Islands Department of Environment, with an agreement that I determine the species identity of the novel introduced population using genetic techniques.

2.2 Green Anoles

Dr. Reynolds collected three samples of *A. fairchildi* from Cay Sal Island, Bahamas in August 2015. He additionally obtained tissues from another green anole species, *A. smaragdinus*, from South Bimini (n=2) and Great Ragged (n=2) islands in the Bahamas (Fig. 1). From these *A. fairchildi*, *A.* s*maragdinus*, and the unknown green anole samples, I extracted whole genomic DNA using the Wizard SV® Kit (Promega, Madison, WI) and subsequently stored the extracts at -20°C. The first extractions of *A. fairchildi* seemed to contain low quality DNA, likely owing to degraded liver samples or the use of too much tissue in the extraction attempts. Therefore, Dr. Reynolds obtained tail-tips of the specimens housed at the Museum of Comparative zoology at Harvard University, and I re-extracted the DNA.

I used PCR to amplify fragments of the mitochondrial genome for *Anolis* tissue samples (NADH subunit 2 [*ND2*]; conditions in [12]). I used two published ND2 5' to 3' primers L4437- AAGCTTTCGGGCCCATACC[13] and H5730-AGCGAATRGAAGCCCGCTGG[5]. I conducted PCR in 22 µl reactions: 10.4 µl H_2O, 5 µl Flexi Buffer, 2.5 µl $MgCl_2$, 1.5 µl dNTPs, 1.25 µl each primer, and 2.25 µl GoTaq Master Mix. The parameters for mtDNA included initial denaturing at 95ºC for 2:30 min, followed by 30 cycles of amplification: denaturing at 95 ºC for 35 s, annealing at 53 ºC for 35 s, and extension at 72 ºC for 2:30 min, then a final extension at 72 ºC for 10 min. I examined PCR products using gel electrophoresis to ensure the reactions produced products in the desired range. I sent PCR products to be purified and sequenced in both directions on an automated sequencer (ABI 3730XL) at the Genomic Sciences Laboratory at North Carolina State University, Raleigh, NC. I assembled contigs and manually verified ambiguous base calls using GENEIOUS® 10.0.1 (Biomatters, Auckland, New Zealand).

To determine the phylogenetic relationship of *A. fairchildi* and the unknown green anoles to other Cuban and Bahamian green anoles, I downloaded an additional 216 ND2 sequences representing *A. carolinensis* clade green anoles (*sensu* [6]) from GenBank (data from [6,8,9]). I then aligned our new and GenBank sequences using the CLUSTALW 2.1[14] algorithm in GENEIOUS using reference sequences and default parameters. I used a GenBank sequence of *A. sagrei* as the outgroup for the phylogeny. I selected the best-fit model of molecular evolution for the *ND2* locus (*HKY+I+G*) using Bayesian information criterion (BIC) in JMODELTEST2v.2.1.10[15,16]. I ran a maximum likelihood (ML) analysis using the RAxML algorithm[17] using the RAxML plugin-in for GENEIOUS. I used the GTRGAMMA model and the rapid bootstrapping algorithm with 1000 bootstrap (BS) replicates followed by the thorough ML search option with 100 independent searches. I consider BS values above 70% to indicate relatively well-supported clades[18]. To estimate divergence times across the mitochondrial gene tree, I constructed a time-calibrated *ND2* tree in the program BEAST v1.8.3[19]. I used a relaxed molecular clock model and a rate of molecular evolution of 0.65% divergence per lineage, per million years, a rate commonly used in other *Anolis* studies[20,21]. I ran the MCMC for 100 million generations using the *HKY+I+G* substitution model, a Yule speciation prior, and an uncorrelated lognormal relaxed (UCLN) molecular clock model. I repeated the analyses three times with different starting numbers for the MCMC chain and all other conditions being the same to assess independent convergence among MCMC analyses. I assured adequate mixing of the chains by calculating the effective sample size (ESS) values for each model parameter, with ESS >200 indicating adequate sampling of the posterior distribution. I examined whether independent runs converged by a comparison of likelihood scores and model parameter estimates in TRACER v1.5[22]. I then combined the results from the three separate analyses using LOGCOMBINER and generated a maximum clade credibility (MCC) tree using TREEANOTATOR.

It is worth noting that in this case, divergent times estimated this method are actually coalescent times in the sense that a single gene coalescent analysis represents the time of coalescence of two lineages and not allopatric divergence. In every case under the coalescent, divergence times will be overestimated relative to divergent events owning to allopatry [23].

2.3 Brown Anoles

On the same 2015 research cruise to the Cay Sal Bank, Dr. Reynolds collected a number of *A. sagrei* from five islands in the region. He selected and sequenced seven representatives from this set using the same ND2 primers and conditions as above in 2015. I cleaned and aligned these sequences as above, then re-aligned them with a large-scale alignment from Dr. Reynolds[24] that contains ~300 representative *A. sagrei* haplotypes from across the range of the species (Bahamas to Central America). I estimated ML and Bayesian phylogenetic trees for the complete brown anole dataset, using the same conditions and priors as for the green anoles.

3. Results

3.1 Origins of Cay Sal Green Anoles

The alignment consisted of 1,138 base pairs of the coding region of ND2 plus tRNA and 226 samples- the three unknown green anoles from Grand Cayman, three *Anolis fairchildi,* and four *A. smaragdinus* from Ragged Island and Bimini and 216 published sequences from GenBank. jModelTest 2.1.8 selected the *HKY+I+G* evolutionary model for the Bayesian and Maximum Likelihood analyses. Both Bayesian and ML analyses produced similar topologies for the green anole tree; hence I show only the Bayesian phylogeny. *Anolis fairchildi* formed a monophyletic clade sister to the west Cuban *A. porcatus*, and not to Bahamian *A. smaragdinus* (Fig. 4). I found evidence for a recent coalescence between *A. fairchildi* and West Cuban *A. porcatus*, though with low support (BS=37; PP=0.73; 95% Highest Posterior Density (HPD) = 0.6 – 1.6 Mya). The root node of *A. fairchildi* has a mean coalescent time of 1.05 Mya.

3.2 Origin of Grand Cayman Green Anoles

The unknown green anoles from Grand Cayman seem to originate from West Cuba (Fig. 4). I found evidence for a recent coalescence between the three unknowns from Grand Cayman (BS = 99; PP=0.99; 95% HPD = 0.62 – 3.45 Mya). The root node for all three unknown green anoles has a mean coalescent time of 2.34 Mya. My analysis suggests that two of the unknown samples form a monophyletic clade sister to the introduced Dominican Republic *A. porcatus* (PP = 1). The other unknown sample forms a clade with *A. porcatus* from West Cuba. These data support a species identification of western Cuba *A. porcatus* for the introduced Grand Cayman population.

3.3 Origin of Cay Sal Brown Anoles

The colonization of the brown anoles showed similar results to *A. fairchildi*. The alignment consisted of 1,098 base pairs of the ND2 coding region plus some tRNA. The alignment contained a total of 298 sequences, including seven new sequences: two *A. sagrei* from Cay Sal Island, one from Elbow Cay, two from Elephant Rocks, and two from Dog Rocks. Cay Sal Bank *A. sagrei* formed a monophyletic clade sister to *A. sagrei* from West Cuba. I found evidence for a recent coalescence between Cay Sal's *A. sagrei* and West Cuban *A. sagrei* (BS = 43; PP = 0.73; 95% HPD = 1.71 – 2.66 Mya). The root node of Cay Sal's *A. sagrei* had a mean coalescent time of 2.16 Mya. I found evidence of an early divergence of *A. sagrei* to Dog Rocks from the northern islands of Cay Sal Bank, Elephant Rock and Elbow Cay with a mean coalescent time of 1.08 Mya (BS = 100; PP = 1; 95% HPD = 0.66 – 1.54 Mya).

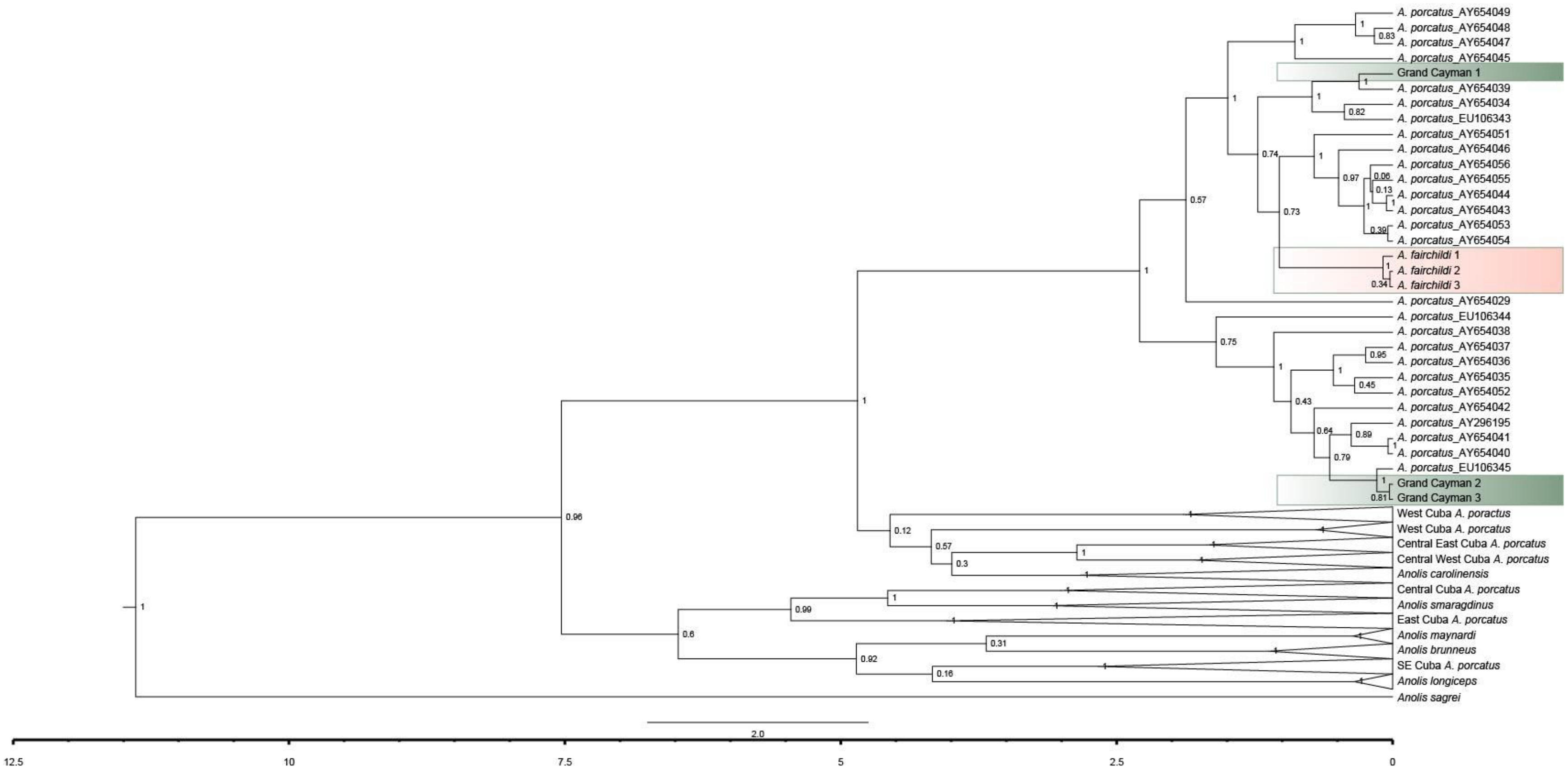

Figure 4. Bayesian phylogeny of Cuban Green Anole species (*carolinensis* clade), with major Cuban clades and sister species collapsed. Red represents *A. fairchildi* from Cay Sal Bank, and green represents the three unknown anoles from Grand Cayman. Numbers at nodes are posterior probabilities. The scale bar at the bottom represents divergence times in Mya.

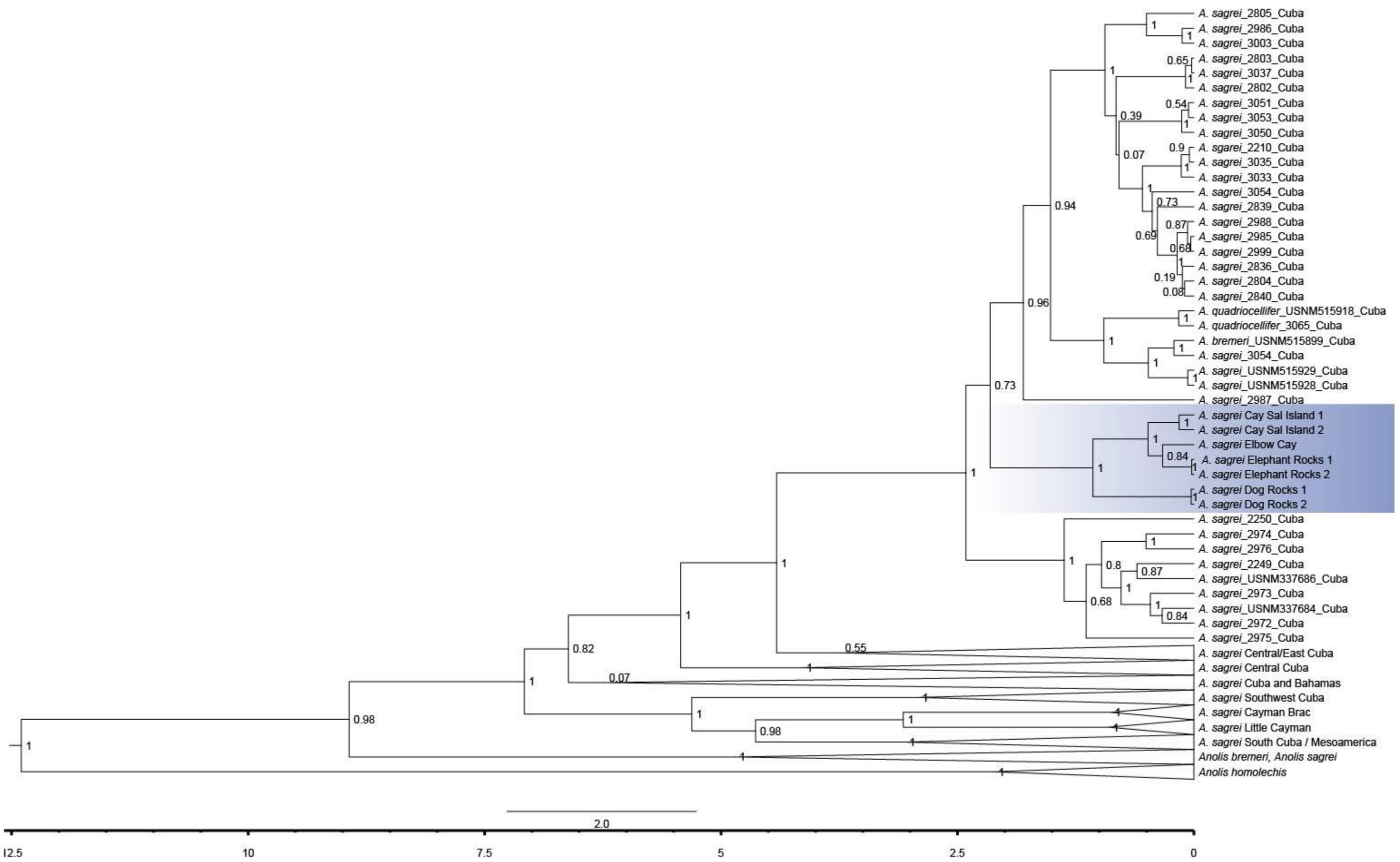

Figure 5. Bayesian phylogeny of the Cuban Brown Anole, *A. sagrei*, complex, with major Cuban and sister species collapsed. Blue represents the *A. sagrei* individuals sampled from islands on Cal Say Bank, and numbers at nodes are posterior probability values. The scale bar at the bottom represents divergence times in Mya.

4. Discussion

4.1 Cay Sal Green Anoles

The genetic data suggest that *A. fairchildi* has recently colonized the Cay Sal Bank, Bahamas from somewhere in western Cuba since the late Pleistocene. All five non-Cuban green anole populations, including *A. brunneus*, *A. carolinensis*, *A. longiceps*, *A. smaragdinus*, and *A. maynardi*, originally dispersed out of Cuba[6]. The Cuban center of origin supports the Bayesian phylogeny for the green anole (Fig. 4). Moreover, my analysis is supported by conclusions of another recent study of a racer snake found on Cay Sal Bank that also originated from western Cuba [25]. Due to the history of the island flooding, it is possible that many of the terrestrial vertebrates that inhabit the island have arrived relatively recently. The currents surrounding Cay Sal suggest that vertebrates travelling from western Cuba via overwater dispersal would potentially be swept to Cay Sal Island.

A. fairchildi is nestled within the *A. porcatus* clade, a species widely distributed across Cuba. Therefore, this colonization of Cay Sal appears to have occurred recently. Although *A. fairchildi* is currently recognized as valid species, my data demonstrate that they do not seem phylogenetically distinct from *A. porcatus*. While I note that the PP is only 0.73, suggesting a basal polytomy of the lineages to *A. fairchildi* and west Cuban *A. porcatus,* the phylogeny still supports the hypothesis that *A. fairchildi* populations are no more than a subspecies of *A. porcatus* under the phylogenetic species concept. The Cuban native, *Anolis porcatus*, has two allopatrically distributed east and west populations that are phylogenetically distinct and deeply divergent [5]. The eastern Cuban *A. porcatus* is more closely related to *A. maynardi* from Little Cayman Island than it is to western Cuban *A. porcatus*[25]. The phylogeny of the Cuban *A. porcatus* complex is ambiguous and in need of revision. Nevertheless, my data clearly indicate that *A. fairchildi* is a Cuban, not a Bahamian, derivative, and furthermore, that its specific recognition makes *A. porcatus* paraphyletic.

4.2 Grand Cayman Green Anoles

Both the ML and Bayesian phylogenies suggest that the unknown Grand Cayman anoles are *A. porcatus* originally from western Cuba. One of the unknown samples is monophyletic with respect to mitochondrial DNA while the other two have a deeper divergence forming a monophyletic clade with the Dominican Republic population of *A. porcatus* (Fig. 4). Due to the majority of *Anolis* lizards being endemic on different Greater Antillean islands, colonization events are rare. Therefore, new populations naturally colonizing the island via overwater dispersal is unlikely. However, due to the deep divergence of the unknown samples, the most likely explanation is that all three came from Florida despite the samples not being *A. carolinensis.* The state of Florida has anole species consistently introduced from across their native ranges, resulting in a mixture of diverse species interacting ecologically and reproductively leading to genetic mixture across lineages. While the results identify the unknown species on Grand Cayman conclusively as *A. porcatus*, I am unable to determine the exact origins of this population.

4.3 Cay Sal Brown Anoles

In the Bayesian phylogeny, the Cuban *A. sagrei* clade has a deep divergence between the eastern and western populations (Fig. 5). The population *of A. sagrei* on Cal Say forms a monophyletic clade within the western Cuba *A. sagrei* population. The phylogenies suggest weak support (PP = 0.73) for reciprocal monophyly of the Cay Sal brown anoles and the sister lineage of Cuban brown anoles, though Cay Sal anoles are nested firmly within Cuban *A. sagrei*. Currently, populations of brown anoles in Cay Sal are recognized as members of the Bahamian brown anole subspecies *A. sagrei ordinatus*. However, these results suggest that *A. sagrei* on Cay Sal originated from Cuba via overwater dispersal, similar to *A. fairchildi*. Importantly, there is a coalescent divergence time of approximately 2 million years ago between the Cuban and Cay Sal *A. sagrei*, suggesting that Cay Sal Brown anoles are a relatively old lineage. Due to the brown anole's earlier colonization of the bank, they inhabit many of the islands including the northern islands, Elbow Cay, Dog Rocks, and Elephant Rocks. The population from Dog Rocks is more divergent than the other populations most likely due to its isolation compared to the other islands.

Similar to the green anole *carolinensis* clade, the *A. sagrei* clade is in need of revising. The *A. sagrei* clade is widespread across the Greater Antillean Islands, resulting in distinct and divergent evolutionary lineages. Some lineages diverged more than 7 million years ago. However, unlike the green anoles from Cuba, *A. sagrei* has retained similar morphological features[24]. Therefore, despite their independent evolutionary lineages, *A. sagrei* has kept similar traits during each colonization event. Although the taxonomy has been debated, some populations are recognized as distinct species including, *A. nelsoni* and *A. luteosignifer*, resulting in the paraphyletic *A. sagrei* clade. Furthermore, *A. sagrei* of Cay Sal should not be recognized as the subspecies *A. sagrei ordinatus*, but due to the current taxonomy, it may not render the qualifications of a distinct species. Further work examining nuclear data is needed to determine how genetically distinct the *A. sagrei* population is on Cay Sal Bank.

5. Conclusion

Anolis lizards have diversified across the Greater Antillean islands resulting in hundreds of species. On the Cay Sal Bank, two key findings regarding *A. sagrei* and *A. fairchildi* were established. First, based on mtDNA, the *Anolis* lizards that inhabit the bank both seem to have originated from western Cuba, which coincides with the current vectors of the channels surrounding the bank. Second, the current understanding that *A. fairchildi* is a distinct species is not valid under the phylogenetic species concept. The phylogeny suggests that *A. fairchildi* is a subgroup nestled in the *A. porcatus* clade. *Anolis sagrei* of Cal Say Bank does not appear to be a member of the subspecies *A. sagrei ordinatus* from the Bahamas. Instead, they appear to be divergent from *A. sagrei* from western Cuba. The results also show that on Grand Cayman, the unknown green anole species appear to be *A. porcatus*, but their origin remains ambiguous owing to serial introductions of this species from Cuba to major shipping ports that communicate with Grand Cayman.

6. Acknowledgements

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