1-31-2019

Using archival DNA to elucidate anole phylogeny

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In 1984, Rusell Higuchi and colleagues (Higuchi et al., 1984) published a path-breaking paper on the quagga, a zebra-like equid from southern Africa that had gone extinct about a century earlier. In the paper they reported that they had obtained DNA from a dried museum skin, and had been able to sequence 229 bp of mitochondrial DNA. The ability to obtain DNA from specimens such as the quagga—preserved, if consciously preserved at all, without any intent to preserve the DNA—soon gave rise to a now flourishing field of study: the study of evolutionary history using "ancient" DNA sequences. The development of the field has not been without hiccups. In some of his earliest studies, on Egyptian mummies, Svante Pääbo, now a leader in ancient DNA studies, turned out to have sequenced modern human contaminants (Pääbo, 2014). But the methodology of sequencing DNA in general, and ancient DNA in particular, has advanced greatly, and has now been successfully applied to a great diversity of extinct taxa, from Vegas Valley leopard frogs (Hekkala et al., 2011) and Bahamian tortoises (Kehlmaier et al., 2017), to Mascarene skinks (Austin and Arnold, 2006) and Tasmanian tigers (White et al., 2018). For DNA obtained from museum specimens that are no more than a century or two old, we prefer the term "archival DNA"—defined as DNA extracted from specimens that were not preserved with the intent of preserving the specimens' DNA—leaving the term "ancient DNA" to refer to the sorts of serendipitous and non-scientific preservation found in much older specimens such as mummies, Neanderthals, and wooly mammoths.

Before new experimental methods can be accepted, they must be validated by showing that new results comport with well-confirmed earlier findings. As Sir Arthur Eddington (1935) quipped, no new experimental finding can be accepted until it is confirmed by theory. Equally important in the case of extinct species, it must be shown that the risks of destructive sampling of irreplaceable specimens are outweighed by the rewards of new and otherwise unobtainable data. Thus, the second figure in Higuchi et al. (1984)—the first was the sequence of A's, G's, C's, and T's themselves—is a phylogenetic tree showing that the quagga, just as was already well-confirmed by morphological data, was indeed a member of the horse family, and nearer to a cow than a human. The exact relations of the quagga have been confirmed and further elucidated by later sequencing work by Leonard et al. (2005), which shows that quaggas are most closely related to plains zebras. As this and other examples show, archival DNA has proven to be a

valuable source of data for the study of extinct species and populations.

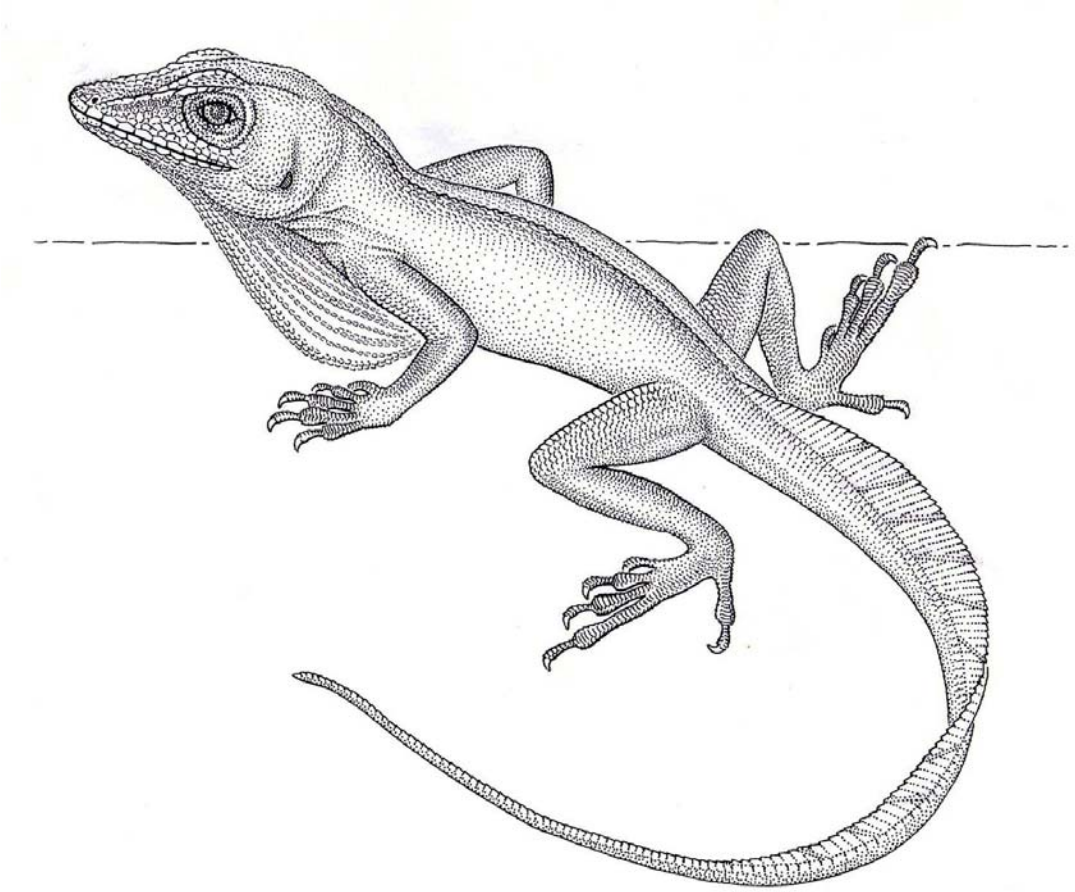

Figure 1. MCZ 36138, the holotype of *Anolis roosevelti*. Laszlo Meszoly, del.

For anoles, we are fortunate in that, of the 400 or so species known from living specimens, there has been little extinction (Böhm et al., 2013). Although some poorly known anole species have not been recently collected, other poorly known species—for example, *Anolis proboscis* (Poe et al. 2012)—have been recently rediscovered and more thoroughly studied. The only species of anole widely acknowledged to be likely to have gone extinct in historical times is *Anolis roosevelti* (Fig. 1), which inhabited the eastern islands of the Puerto Rican Bank, where it is known to have occurred on Vieques, Culebra, St. John, and Tortola (Fig. 2). Based on the reports obtained by Chapman Grant (1931, 1932), the species' describer, and its morphology, *roosevelti* has been interpreted as the crown-giant ecomorph of the eastern Puerto Rican Bank, where it would have been the ecological vicar of *Anolis cuvieri* of the Puerto Rican main (Fig. 3; Mayer, 1989). Last collected on Culebra in 1932, a number of searches in its known range, most notably heroic endeavors by Ava Gaa Ojeda Kessler (2010), in and around its last known haunts on Culebra, have turned up nothing; and though we still hold out some hope for its survival, especially in the still little explored former naval reservation on eastern Vieques, the species is

usually considered extinct.

Figure 2. Puerto Rico and the Virgin Islands, showing the known distribution of *Anolis roosevelti* (stars). From west to east, the islands are Vieques, Culebra, St. John, and Tortola (north at top). Base map: Google Earth.

With no fresh specimens available, phylogenetic studies of *roosevelti* have necessarily been morphology-based. Using the morphological characters then available—primarily Richard Etheridge's (1959) osteological characters—Ernest Williams (1972) attempted to place *roosevelti* in a phylogenetic context amongst the other Puerto Rican anoles. Osteologically, *roosevelti* is an alpha-anole (lacking transverse processes on the caudal vertebrae), with an arrow-shaped interclavicle, and three fixed and two free inscriptional ribs. This places it near the base of the tree constructed by Williams, but the overall evidence is not strong.

Liam Revell, Luke Mahler, Graham Reynolds, and Graham Slater (2015) tried a novel method using metric characters to infer *roosevelti's* relationships. They found it to be near the Cuban crown giants, not the Puerto Rican giant, *cuvieri*. But for some time the standard for phylogenetic estimation in anoles has been DNA sequence data. With no recently collected specimens, the only option for study of *roosevelti* is archival DNA. As noted earlier, in the case of extinct species, it must be shown that the risks of destructive sampling of irreplaceable specimens are outweighed by the rewards of new and otherwise unobtainable data. This consideration is clearly of concern with *roosevelti*, since only six extant specimens are known: four collected by A.H. Riise in the 1860s, and two by Chapman Grant in the 1930s. An additional consideration is that it is very difficult to get DNA from specimens fixed in formalin, and since Stejneger's promotion of formalin as a fixative in 1911, most collectors have used it. Riise's specimens, collected in the 1860s, would be more likely to have been fixed in ethanol. (Not to mention that Riise founded what is now the largest liquor store in the Virgin Islands, and

so seems to have had an affinity for alcohol!)

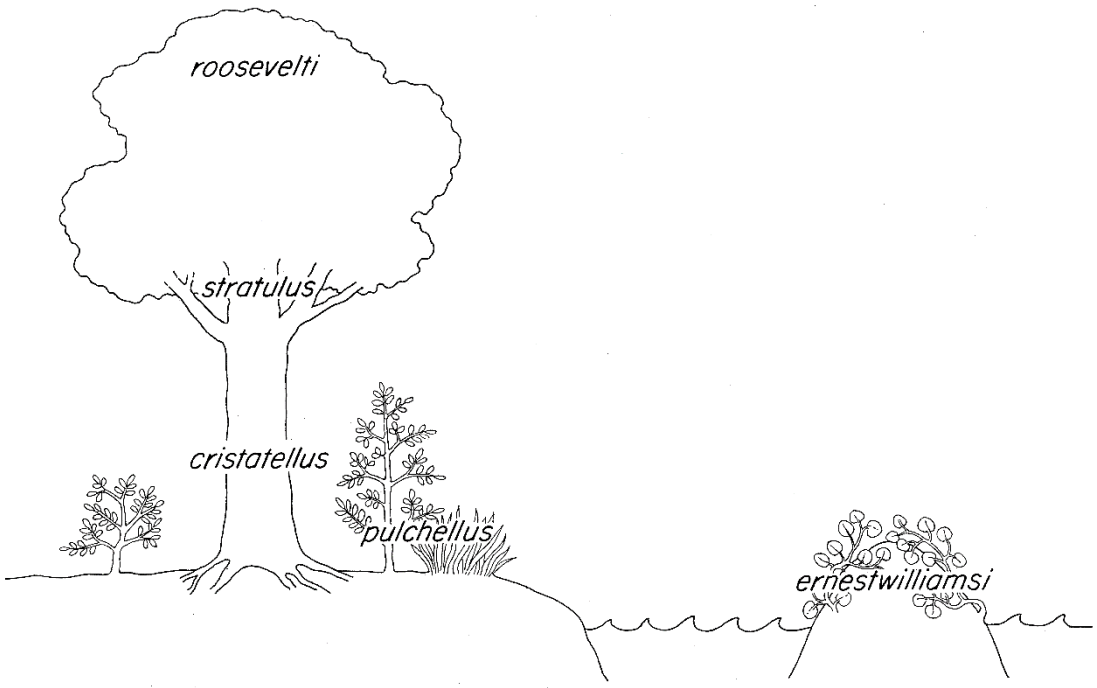

Figure 3. Ecological distribution of the anoles of the eastern Puerto Rican Bank (Virgin and Passage Islands). *Anolis roosevelti* is the crown-giant ecomorph, *A. stratulus* is the trunk-crown ecomorph, *A. cristatellus* is the trunk-ground ecomorph, and *A. pulchellus* is the grass-bush ecomorph. *A. ernestwilliamsi* is a *cristatellus* derivative endemic to the largely *Coccoloba*-covered Carrot Rock. Laszlo Meszoly, del.

So, preliminary to study of *roosevelti*, we have attempted the extraction of archival DNA from specimens of *Anolis cristatellus*, which is abundant and distributed throughout the Puerto Rican Bank—literally from one end to the other— and with close relatives on off-lying island banks. By showing that we can extract and sequence archival DNA from this species, and that the results obtained comport with what is known about this well-studies species, we can pass the "Eddington test", and thus have greater justification in consumptively sampling from the irreplaceable *roosevelti* specimens, and greater confidence in the results of that sampling.

Methods

Our goal was to utilize specimens that are as similar as possible in their history to the extant specimens of *roosevelti*. We have studied three specimens of *cristatellus* collected on Vieques by Riise in about 1861, five specimens collected on Vieques and Culebra by Grant in 1931, three more recent specimens collected by Skip Lazell in the Virgins in 2000, and one specimen collected by one of us (TG) in Puerto Rico in 2014. This last one, unlike the others, was fixed in ethanol with the intent to preserve its DNA. For archival DNA we thus have eight

specimens of *cristatellus* collected approximately coincident in time and place with Riise and Grant's specimens of *roosevelti*, plus three more recent ones; as well as a single 'modern' specimen (Table 1).

Table 1. Specimens used and the results of DNA extraction and sequencing.

Specimen	Year	Locality	fixative	DNA (ng/ml)	Library	mtDNA assembly	ND2 phylogeny
ZMUC R37381	1861	Vieques	ethanol	<50	good	fail	no
ZMUC R37383	1861	Vieques	ethanol	<50	good	good	yes
ZMUC R37386	1861	Vieques	ethanol	<50	good	good	yes
MCZ R35732	1931	Vieques	ethanol	68	good	partial	yes
MCZ R35735	1931	Vieques	ethanol	145	good	good	yes
MCZ R35739	1931	Vieques	ethanol	55	good	good	yes
MCZ R35953	1931	Culebra	ethanol	54	poor	fail	no
MCZ R35959	1931	Culebra	ethanol	<50	poor	fail	no
MCZ Z28485	2000	Necker Id.	isopropanol	416	good	partial	yes
MCZ Z28486	2000	Necker Id.	isopropanol	385	good	good	yes
MCZ Z28585	2000	Tortola	isopropanol	267	good	partial	yes
TG 2223	2014	Puerto Rico	ethanol	520	good	good	yes

We expected that Riise's three *cristatellus* from the Zoological Musuem in Copenhagen (ZMUC), collected about 1861, would have been fixed in ethanol, and we can confirm this, as they all had the opaque white pupils characteristic of ethanol fixation (Fig. 4; Simmons, 2014). Unexpectedly, Grant's specimens were also ethanol fixed, as shown by their also having opaque, white pupils, as kindly confirmed for us by Jose Rosado. Skip Lazell's specimens (MCZ Z numbers in Table 1) were fixed primarily in isopropanol (which is available by retail sale throughout the West Indies). For the cataloged Museum of Comparative Zoology (MCZ) specimens (MCZ R numbers in Table 1), Breda Zimkus took thigh muscle and liver tissue for us; for the others, we took liver tissue from a ventral incision, little different from that made in a fresh specimen from which tissue is taken (Fig. 5).

Figure 4. ZMUC R 37381, *Anolis cristatellus*, showing the opaque white pupil indicative of ethanol fixation.

Figure 5. ZMUC R 37383, *Anolis cristatellus*, showing the ventral incision for removal of liver tissue.

DNA was extracted from the tissues following the protocol of Ruane & Austin (2017). llumina libraries were prepared using NEBNext Paired-end library kit, and sequenced with Illumina HiSeq 2500 at the Medical College of Wisconsin. Reads were cleaned and trimmed, and PCR duplicates removed. *De novo* assembly was performed using CLC Genomics Workbench for whole mitogenome assembly. Reads were mapped to assembled *A. cristatellus* mitogenome using Geneious for partial mitogenome assembly. The mitogenome was annotated using mitoAnnotator. We performed two phylogenetic analyses, one using whole mitogenomes with our archival and modern DNA, plus multiple anole taxa, including *cristatellus*, from Gen Bank; and a second phylogenetic analysis using just ND2. Alignment was done with MUSCLE (using data from Reynolds at al., 2017 for ND2), and trees estimated using RaxML.

Results and Discussion

We obtained quantifiable DNA from 8 of 12 samples (detection limit 50 ng/ml), and good Illumina libraries from 10 of 12 samples. *De novo* assembly produced complete or near complete (>80% complete) mitogenomes in 6 of 12 samples, and partial mitogenomes from 3 of the 6 remaining samples (Table 1). Unfortunately, sequences could not be recovered from either of the Culebra samples, both of which were collected by Grant.

The assembled whole mitogenome (Fig. 6) appears as would be expected, with a fairly typical genome size and arrangement of the genes, with the exception of ATPase 8, which is found at about 16.2 kb; normally it's at about 8.5 kb, near ATPase 6.

The mitogenome phylogenetic analysis was designed to demonstrate that the archival sequences were what would be expected of *cristatellus*, and utilized a number of other anole mitogenomes, either from GenBank or generated in TG's lab. The results (Fig. 7) clearly show that the archival *cristatellus* DNA samples—shown boxed in gray—form a clade with the two modern samples of *cristatellus*— ours (TG 2223), and another from GenBank. Note that several of the archival samples form their own subclade, but that another is within the adjacent, otherwise modern, subclade of *cristatellus*. This result confirms that the archival DNA is indeed *Anolis cristatellus* DNA.

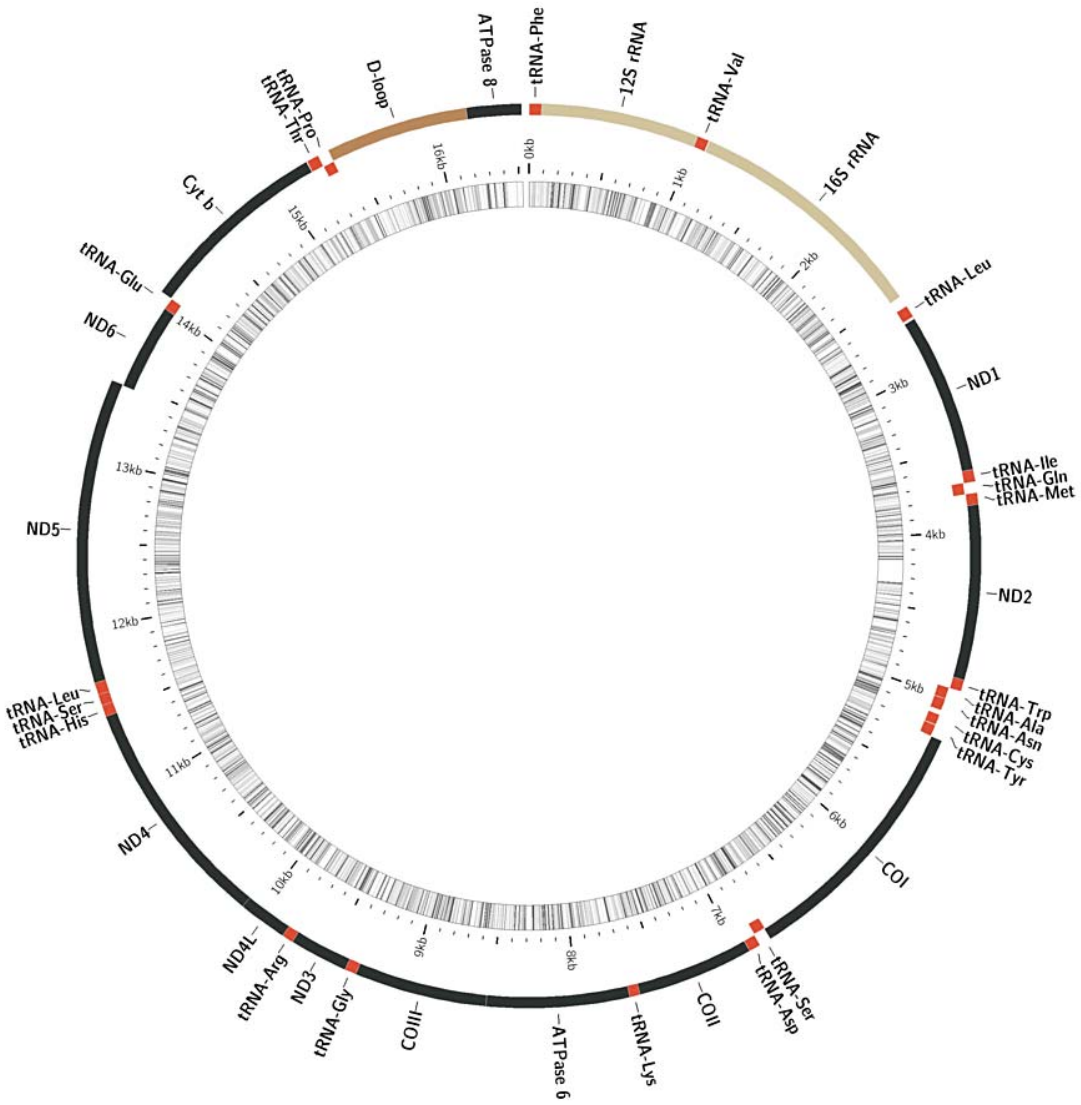

Figure 6. Annotated mitogenome of *Anolis cristatellus*.

The ND2 phylogenetic analysis was designed to look at the placement of the archival samples within *cristatellus*, and utilized a large number of sequences of *cristatellus* and its close relatives, most from Graham Reynolds and colleagues' recent paper (2017). There are three things to note in the estimated phylogeny (Fig. 8). First, Skip Lazell's British Virgin Island specimens (MCZ Z numbers) fall amongst other British Virgin Island samples, as expected. Second, all the archival Vieques samples (MCZ R numbers) are either sister to another Vieques sample, nested within a Vieques clade, or nested within a clade that includes Vieques specimens.

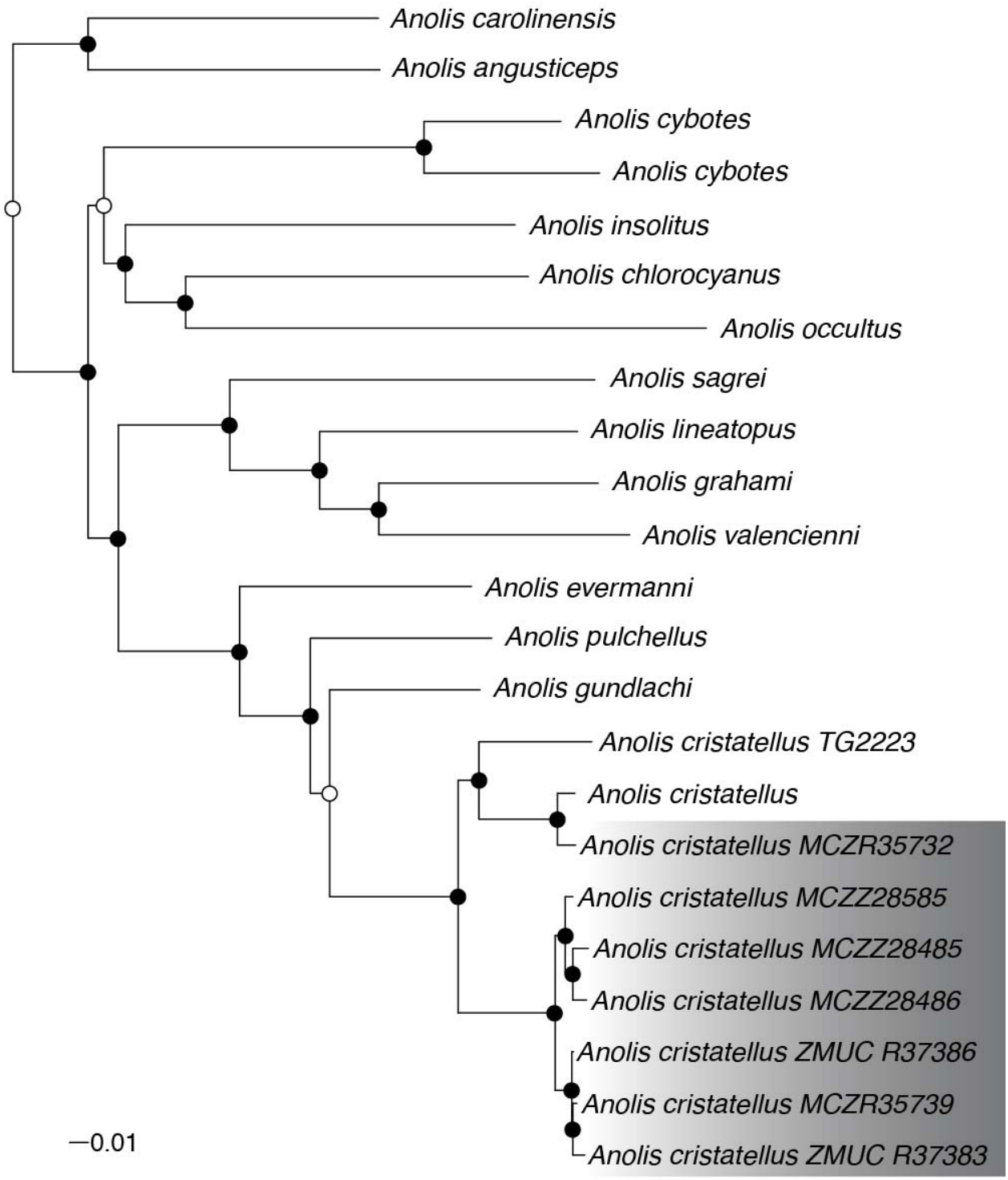

Figure 7. Phylogeny estimated from mitogenome sequences with RaxML. Black circles at nodes indicate a bootstrap percentage greater than 70. Seven archival samples are highlighted. These samples and the modern sample TG2223 are from this study; other samples from GenBank or generated in Gamble lab.

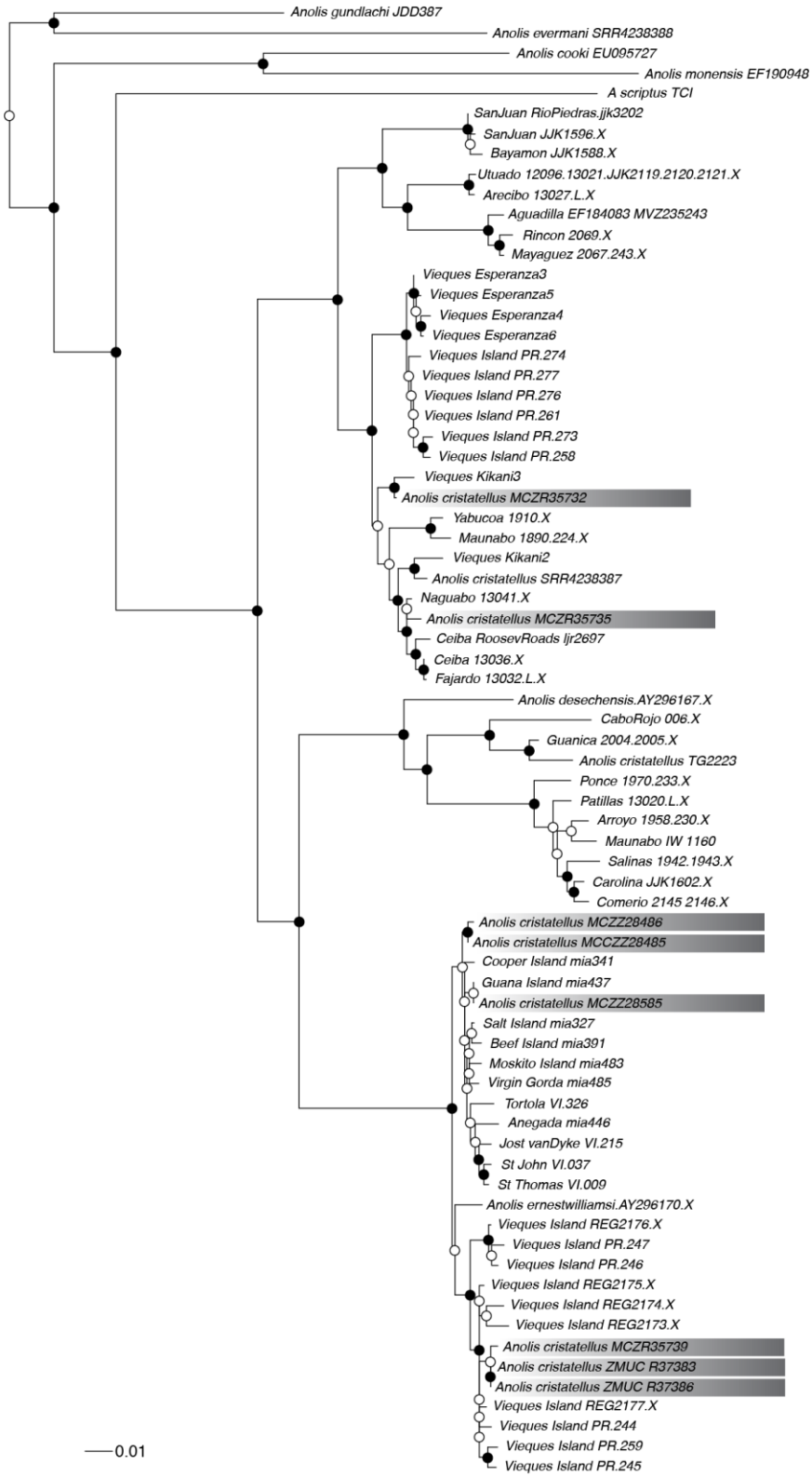

Figure 8. Phylogeny estimated from ND2 sequences with RaxML. Black circles at nodes indicate a bootstrap percentage greater than 70. Eight archival samples are highlighted. These samples and the modern sample TG2223 from this study; other samples mostly from Reynolds et al. (2017).

Also note that Grant's specimens are divided between the two divergent clades identified by Reynolds et al. (2017)—"PR East" and "Virgin Islands"; both of Riise's specimens are in the "Virgin Islands" clade. And finally, our modern specimen, from Boqueron, Puerto Rico, falls within a clade of other southwestern Puerto Rico specimens. All these results are as would be expected. Together, they confirm the localization of the samples to geographically sensible parts of the tree.

Conclusion

So, archival DNA in anoles has passed the Eddington test—it produces results that are entirely reasonable given what we already know, based on well-confirmed estimates of phylogeny. We thus conclude that extraction and analysis of archival DNA is a promising method for investigations of *Anolis roosevelti.* The results for *roosevelti*, unlike those for *cristatellus*, for which we already had well-confirmed expectations, will be novel and interesting. And we also conclude that archival DNA has promise for investigations of the genomes and phylogeny of anoles in general.

Acknowledgments

We are very grateful to the museum curators who permitted us to use tissue from historically important specimens, and recognized the logic of working with the more numerous specimens of *Anolis cristatellus* before moving on to the irreplaceable specimens of *roosevelti*. We give our heartfelt thanks to Daniel Klingberg Johansson and Peter Rask Møller at the Zoological Museum, University of Copenhagen, and to
Jonathan Losos, Jose Rosado, James Hanken, and Breda Marie Zimkus at the Museum of Comparative Zoology. James D. 'Skip' Lazell generously provided specimens for our use. Alan Resetar at the Field Museum of Natural History kindly received loans, and provided working space for us to take tissue. Funding was provided by Marquette University and by NSF DEB1657662 to TG.

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