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Complete Protein Coding Mitochondrial Genomes of 13 West Indian Boas

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Abstract

Mitochondrial DNA (mtDNA) have long been used as a tool for genetic analyses, with most studies relying on sequence data from only one or two protein-coding regions, generally amounting to <2,000 base pairs of sequence data. The development of next-generation sequencing methods over the last decade has enabled the generation of large amounts of sequence data for an increasingly lower cost per base. These methods typically target nuclear DNA, but because of mtDNA's abundance in most eukaryotic cells, mitochondrial regions are often sequenced as well. When indirect, or "off-target" mtDNA is obtained, it is often referred to as by-catch, implying that its inclusion in sequencing output was not the intention of the sequencing experiment. Because next-generation sequencing methods generate so many sequences per run (millions), by-catch regions can amount to substantial numbers of sequences and thus might prove useful. Indeed, many researchers now value these by-catch regions, particularly because they often allow the characterization of entire mitochondrial genomes (mitogenomes). It is possible to scale this up to simultaneously characterize mitogenomes of entire genera at a time. In this paper, sequence data from a related project was used to simultaneously identify, assemble, and annotate mitogenomes from an entire genus of boid snakes from the Caribbean (genus *Chilabothrus*). To date, no other study has attempted this for boas. Using millions of sequence reads generated from a targeted-capture sequencing project to search for mitochondrial by-catch, mitogenomes were assembled by comparison to known mitogenomes from GenBank. Then, mitogenomes were aligned and annotated to identify and label different gene regions. Finally, a series of protein-coding molecular phylogenies of the entire genus were generated using Bayesian and Maximum Likelihood methods. This study illustrates the feasibility of using sequencing by-catch to simultaneously assemble and annotate mitogenomes from large taxonomic groups, including an entire genus of snakes.

1. Introduction

Mitochondrial DNA (mtDNA) sequences have long proven a useful tool for studies ranging across scales from population genetic to phylogeographic to phylogenetic studies[1,2]. Nevertheless, most analyses of mtDNA have relied upon sequence data from one or two protein-coding regions, amounting to less than 2,000 base pairs (bp) of sequence data. The advent of so-called "next-generation" sequencing methods around the year 2006 have facilitated the generation of orders-of-magnitude more sequence data for an increasingly lower per-base cost. These data typically come from the nuclear genome, as next-generation sequencing libraries are frequently prepared from 200–500 bp fragments of nuclear DNA. Because mtDNA is so abundant in most eukaryotic cells, mitochondrial regions are often sequenced during these processes. These sequences, frequently referred to as mitochondrial by-catch or off-target sequences, can actually prove highly useful even if they are not the intended targets of sequencing experiments. This by-catch can even be used to assemble entire mitogenomes from a single individual, even if the mitogenome was not the intended target of library preparation and sequencing[3]. Hence, there are applications of using next-generation sequencing methods to assemble mitogenomes, even extending to sequencing museum-preserved "ancient" specimens. Using natural history collections is an important aspect of studying biodiversity, specifically in species encroaching towards extinction[4], and thus this technique could even extend to preserved, or rare samples.

Understanding relationships inferred from mitochondrial DNA in addition to targeted nuclear DNA among individuals is a key benefit of certain next-generation sequencing approaches. Such techniques could even help resolve unusual mitochondrial genome structure, such as that of the tuatara (*Sphenodon punctatus*), a lepidosaurian reptile which was recently discovered to contain two entirely separate mitogenomes within its somatic cells, separated by the equivalent of millions of years of mitochondrial evolution[5].

One approach for library-building and next-generation sequencing that tends to yield high levels of mitochondrial by-catch is the method called ultraconserved elements enrichment (UCE). Ultraconserved elements are regions of the genome that are highly similar between distantly related species, such as across tetrapods, and have become an important and relatively inexpensive method to deploy for molecular phylogenetic studies[6]. This approach has gained popularity because of its low cost and high efficiency, and it has been particularly useful for species with few genomic resources available. These conserved elements are largely intron regions of the nuclear genome (although some exons are targeted) and are identified by aligning whole genomes of relevant taxa and identifying invariable sites. Primers, or baits, are then designed around these sites, such that when annealed to whole genomic DNA and subjected to polymerase chain reaction, the library becomes enriched for these loci. Individuals are then barcoded for later identification, pooled into a metalibrary, and sequenced collectively on a next-generation platform, usually an Illumina® HiSeq instrument with paired-end chemistry. Sequencing reactions generally cover not only the target region, but also out into the flanks of the target and even non-target regions (frequently including mtDNA regions)[2]. Sequences are then demultiplexed, or assembled back into individual-based groups based on barcodes, using bioinformatics programs. UCE regions of resulting sequence data are then identified and extracted. At this stage, candidate mitochondrial sequences can be extracted in silico and aligned to known mitogenomes, then assembled into *de novo* mitogenomes, often with high coverage[7].

West Indian boas are a completely insular genus of neotropical boids (Family Boidae) of the genus *Chilabothrus* that dates back to the Miocene. All thirteen currently recognized species occur on the four Greater Antillean Island banks, Isla de Mona, and on the Lucayan Archipelago. Nearly half of these species are newly described or recently elevated species[8]. These boas are nocturnal foragers ranging in body sizes from <1 m to ~4 m, with smaller species typically being ecological specialists and larger species typically being ecological generalists. Generalists occupy arboreal and terrestrial habitats and feed on a wide variety of prey items, while specialists occupy either an arboreal or terrestrial habitat and feed mainly on *Anolis* lizards. The morphology of *Chilabothrus* snakes is influenced by their degree of ecological specialization[9,10]. Several species within this genus face serious conservation concerns such as introduced predators, anthropogenic habitat destruction, poaching for the pet trade, and low genetic diversity[9,11,12,13]. Some of these species are restricted to specific habitat types, which creates a conservation concern for how habitat loss will affect these species[14]. Two species are listed as endangered on the IUCN Red List, while the newly-discovered *C. argentum* is critically endangered and represented by as few as 135 remaining individuals (Figures 1,2).

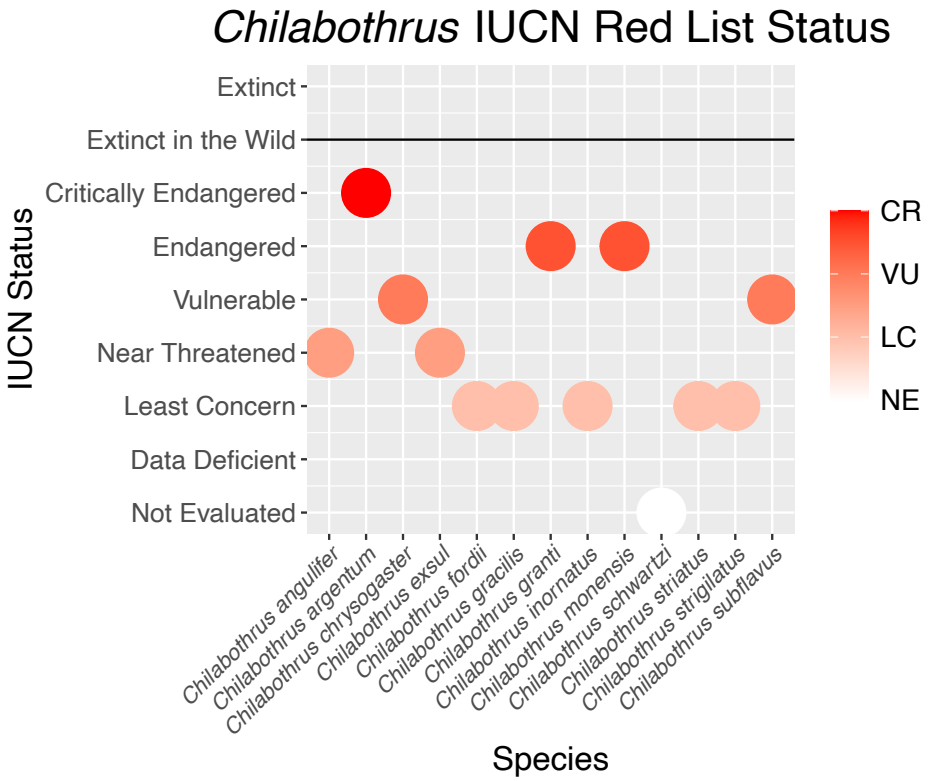

Figure 1. IUCN Red List status of the *Chilabothrus* species analyzed in this study[15].

Figure 1 This illustrates the conservation concerns for the genus as a whole. Legend abbreviations used are CR, critically endangered; VU, vulnerable; LC, least concern; and NE, not evaluated.

Figure 2. A Conception Bank Silver Boa (*Chilabothrus argentum*), the world's most endangered boa. This adult female is one of only 135 adult animals remaining in the entire species. Photograph by R. Graham Reynolds.

Sequencing mitogenomes for threatened and endangered species is important in aiding conservation efforts by providing genomic resources. These data can be useful for inferring relationships among closely and distantly related species as well as for determining important population information such as genetic diversity or population structure. Providing these genetic resources for a threatened genus, such as *Chilabothrus*, will provide crucial information that can be used in studies focusing on their evolutionary relationships as well as those investigating population structure and diversity. In particular, the Conception Bank Silver Boa, *Chilabothrus argentum* (Figure 2), is critically endangered, and being able to provide mitogenomic resources for this species will aid ongoing conservation efforts. Mitochondrial genome sequencing has been a major focus for many endangered species ranging from *Eumenis autonoe*, an endangered species of butterfly in the family Nymphalidae[16], to *Bubalus arnee*, the endangered wild water buffalo[17].

I was able to identify, extract, assemble, and align mtDNA from datafiles containing hundreds of thousands of UCE reads generated for West Indian Boas. I then annotated genomic regions of the mtDNA with reference to a known genome. I found that West Indian boas exhibit a gene order and composition identical to that of other previously characterized boid species. The mitochondrial genomes of boas in this genus consist of 22 tRNAs, 2 rRNAs, 13 protein-coding genes, an origin of the light strand replication (O_L), and two control regions (CR1 and CR2). Here, I present the complete protein-coding mitochondrial genomes for 13 West Indian boa species, as well as additional mitogenome characteristics such as tRNAs and control regions. I was further able to use these mitogenomes to infer well-supported Maximum Likelihood and Bayesian phylogenetic trees. This is the first study to characterize and analyze mitogenomes for an entire genus of boid snakes.

2. Methodology

2.1 Sample collection and DNA sequencing

Tissue samples for *Chilabothrus angulifer, C. argentum* (Figure 2)*, C. chrysogaster chrysogaster, C. exsul, C. fordii, C. gracilis, C. granti, C. inornatus, C. monensis, C. schwartzi, C. striatus exagistus, C. strigilatus, and C. subflavus* were collected by Dr. Graham Reynolds over the last 12 years from wild populations throughout the Caribbean region. Whole genomic DNA was extracted using the Wizard SV® Kit (Promega, Madison, WI) and extracts were subsequently stored at -20°C. The quality of each extraction was examined using a gel electrophoresis check of fragment lengths, followed by fluorometric quantitation of each extraction using a Qubit 3.0® (Thermo Fisher Scientific, Waltham, MA) with the dsDNA BR Assay Kit. Yields above 2.5ng/µl were sent to RAPiD GENOMICS® LLC (Gainesville, FL) for ultraconserved elements (UCE) enrichment and sequence capture using the myBaits® UCE Tetrapods 5kv1 primer set followed by 150 base pair (bp) paired-end sequencing on a single-lane Illumina® HiSeq 2500 run. Libraries were pooled following barcode adapter ligation prior to sequencing, and raw reads consisted of 150 bp-long *.fastq* files.

Multiple individuals were UCE-sequenced for some species, but I only proceeded with one individual per species for subsequent analyses to avoid making chimeric mitogenomes that might complicate future projects.

2.2 Mitogenome assembly

Raw *.fastq* sequence reads were processed using the Linux *phyluce* bioinformatics pipeline described in prior studies[18,19,20]. This toolkit is comprised of conda-packaged Python scripts to facilitate UCE identification and extraction[20]. The Illumiprocessor Python script[19] was used, which calls Trimmomatic[21,22] to trim adapters and low-quality sequences reads. Contigs were then assembled from the cleaned reads using the Velvet algorithm[23] with *kmer* = 51. Bioinformatics were conducted on the CHILABOTHRUS server at the University of North Carolina Asheville, which is a Dell® (Dell Inc., Round Rock, TX) PowerEdge© R420 Server (64c, 128gb RAM) running Ubuntu 21.04.5.

To identify mitochondrial reads, I mapped assembled contigs from the Velvet algorithm to the reference mitochondrial genome of *Boa constrictor* (GenBank ID AB177354)[24] in GENEIOUS 10.2.5 (Biomatters® Auckland, New Zealand) using the GENEIOUS mapper with medium sensitivity. I examined alignments by eye, adjusting individual regions by hand and checking for premature stop codons in protein-coding regions by translation into amino acid codons.

I then used the MITOS2[25] webserver (http://mitos2.bioinf.uni-leipzig.de/index.py) to annotate the consensus sequence for each species of boa, and exported the predicted annotations as .bed files. I loaded these .bed files into the GENEIOUS folder containing mitogenome alignments, which allowed visualization of these predicted annotations

directly on the individual sequences. I then checked each mitogenome and subsequent annotations by eye, making adjustments to annotations to correspond to start and stop codons and known gene regions from the *Boa constrictor* mitogenome available from GenBank (AB177354). Next, I excised non-protein coding regions and used the MAFFT algorithm[26] to generate an alignment of only protein-coding loci from the mitogenomes. These consisted of an average of 10,595 bp of sequence data (range 5,837-11,318 bp per taxon; Table 1).

2.3 Phylogenetic analyses

Using the alignment of protein-coding loci from the mitogenomes, I conducted Maximum Likelihood analysis using the RaxML algorithm implemented in GENEIOUS. I used the GTRGAMMA model where invariant sites are accounted for, as the use of invariant sites parameter (I) has been recommended against in RAxML[27]. I used the rapid bootstrapping algorithm with 10^3 bootstrap (BS) replicates followed by the thorough ML search option with 100 independent searches. I consider BS values above 95% to indicate well-supported clades and those above 70% to indicate moderately-supported clades[28,29].

To estimate divergence times across the mitochondrial gene tree, I inferred a time-calibrated coalescent tree in the program BEAST v1.10.2[30] implementing the BEAGLE library v3.0.1[31] to speed up computations. As in previous studies[12,32], I estimated a substitution rate for the mtDNA locus from the alignment of West Indian boas by constraining the root node of *Chilabothrus* using a normal prior with a mean of 21.7 million years ago (Mya) and a standard deviation of 1.8 Mya. These values were derived from a fossil-calibrated divergence time analysis of the larger Neotropical boid phylogeny[8]. I ran the MCMC for 100 million generations using a Yule speciation prior and an uncorrelated lognormal relaxed clock model. I repeated the analyses three times with different starting parameter values, sampling every 1,000 generations and discarding the first 1,000 trees as burn-in, to generate effective sample sizes (ESS) larger than 200 for all parameters. I assessed convergence of the independent runs by a comparison of likelihood scores and model parameter estimates in TRACER v1.5[33]. I combined results from the three analyses using LOGCOMBINER v1.8, generated a maximum clade credibility tree using TREEANOTATOR V1.8 AND FIGTREE 1.4.4[34].

Table 1. *Chilabothrus* species analyzed in this study and their assembled mitochondrial genome lengths. Included are the total mitogenome lengths as well as the lengths for only protein-coding loci. Note that there were fewer sequence data generated as by-catch for the species *Chilabothrus gracilis*; hence, its mitogenome is less complete.

		mtDNA length (bp)	
Scientific Name	Common Name	Total	Protein-Coding regions
Chilabothrus angulifer	Cuban Boa	16,127	11,289
Chilabothrus argentum	Conception Bank Silver Boa	17,706	11,318
Chilabothrus chrysogaster	Turks and Caicos Boa	16,327	11,284
Chilabothrus exsul	Abaco Islands Boa	15,855	11,197
Chilabothrus fordii	Haitian Ground Boa	15,977	11,078
Chilabothrus gracilis	Haitian Gracile Boa	8,854	5,837
Chilabothrus granti	Virgin Islands Treeboa	15,952	11,304
Chilabothrus inornatus	Puerto Rican Boa	16,218	11,308
Chilabothrus monensis	Mona Island Boa	14,753	10,157
Chilabothrus schwartzi	Crooked-Acklins Boa	16,075	11,307
Chilabothrus striatus exagistus	Hispaniolan Boa	13,857	9,477
Chilabothrus strigilatus	Bahamian Boa	16,331	11,307
Chilabothrus subflavus	Jamaican Boa	15,588	10,866
Boa constrictor (reference)	Boa Constrictor	18,905	11,295

3. Results

3.1 Mitochondrial genomes

For each species, the complete protein-coding mitogenomes were assembled, in addition to most other mitogenome characteristics such as control regions and tRNAs. The total length of the protein-coding loci ranged from 5,837–

11,318 bp per species, with an average of 10,595 bp (Table 1). The overall base compositions for each species are presented in Table 2. These mitochondrial genomes are identical in composition to previously described boid snakes with 13 protein-coding genes, 22 tRNAs, 2 rRNAs, 2 control regions (CR1 and CR2), and an origin of the light strand replication (O_L; Figure 3)[24]. There are no differences in the order of the 13 protein-coding genes and the gene order displayed by all species evaluated is presented in Figure 3.

Table 2. Base composition for each species from the complete protein-coding genomes.

Species	A (%)	T (%)	C (%)	G (%)
C. angulifer	37.2	28.2	23.8	10.8
C. argentum	36.6	26.5	25.5	11.4
C. chrysogaster	36.5	26.3	25.8	11.4
C. exsul	36.5	26.6	25.6	11.4
C. fordii	36.6	25.9	25.9	11.6
C. gracilis	37.1	25.2	26.2	11.5
C. granti	37.0	26.7	25.2	11.1
C. inornatus	36.8	27.4	24.7	11.0
C. monensis	37.6	26.8	24.8	10.8
C. schwartzi	36.6	26.7	25.4	11.3
C. striatus exagistus	36.7	26.2	26.0	11.1
C. strigilatus	36.6	26.3	25.8	11.4
C. subflavus	36.6	25.0	27.1	11.3
Boa constrictor (reference)	36.3	23.7	27.4	12.6

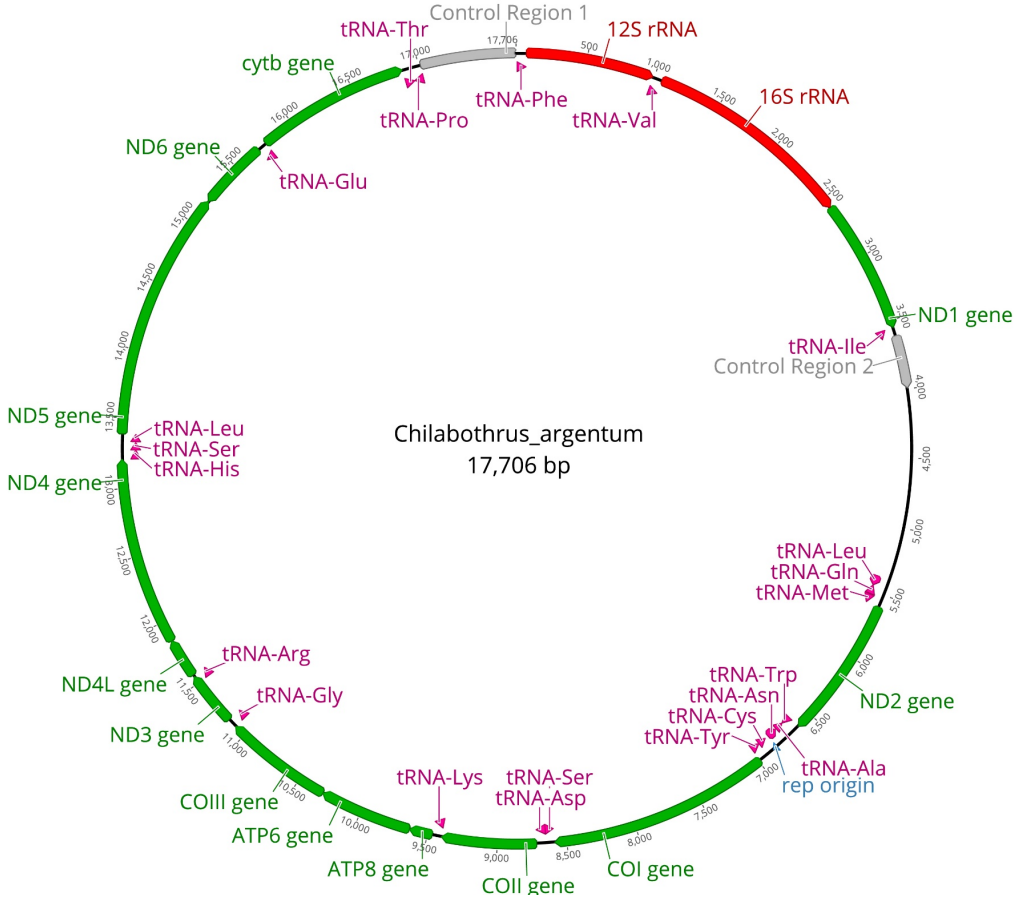

Figure 3. Representative circular mitochondrial genome for *Chilabothrus* snakes.

Figure 3 The mitogenome displayed is from the Silver Boa, *Chilabothrus argentum*. Identical gene order is displayed in all *Chilabothrus* species. Gene abbreviations used are 12S rRNA, small ribosomal subunit; 16S rRNA, large ribosomal subunit; tRNA with three letter amino acid code; ND1-6, NADH dehydrogenase subunits 1-6; rep origin, light strand replication origin; COI-III, cytochrome oxidase subunits I-III; ATP6 and ATP8, ATPase subunits 6 and 8; and cytb, cytochrome *b*.

3.2 Phylogenetic analyses

I aligned the protein-coding loci (10,595 bp) from 13 *Chilabothrus* species and 1 outgroup clade containing: *Boa constrictor* (from GenBank: AB177354), *Boa constrictor* from Puerto Rico (an introduced population), *Boa nebulosa* from Dominica in the Lesser Antilles. The *Boa constrictor* and *Boa nebulosa* were collected by Dr. Graham Reynolds. My maximum-likelihood phylogeny produced a topology with strong support at most nodes (Figure 4). In concordance with previous studies[14], I found that the newly-discovered *C. argentum* is sister to recently rediscovered *C. schwartzi* (BS=100; Figure 4). My topology shows that *C. exsul* and *C. strigilatus* are sister to one another with strong support (BS = 99; Figure 4). This differs from previous topological arrangements using only the mitochondrial CYTB locus (~1100 bp) showing *C. exsul* more closely related to *C. striatus* and the clade containing *C. argentum* and *C. schwartzi*[14]. All other relationships agree with previously presented phylogenetic analyses. The node containing *C. subflavus*, *C. fordii*, and *C. gracilis* is moderately supported (81; Figure 4). While most nodes are strongly supported, the node containing *C. argentum*, *C. schwartzi*, *C. strigilatus*, and *C. exsul* has low support (BS = 44; Figure 4). My maximum clade credibility tree produced the same topology as the maximum-likelihood tree (Figure 5). In addition to confirming the relationships among these taxa, this analysis presents divergence times of these species.

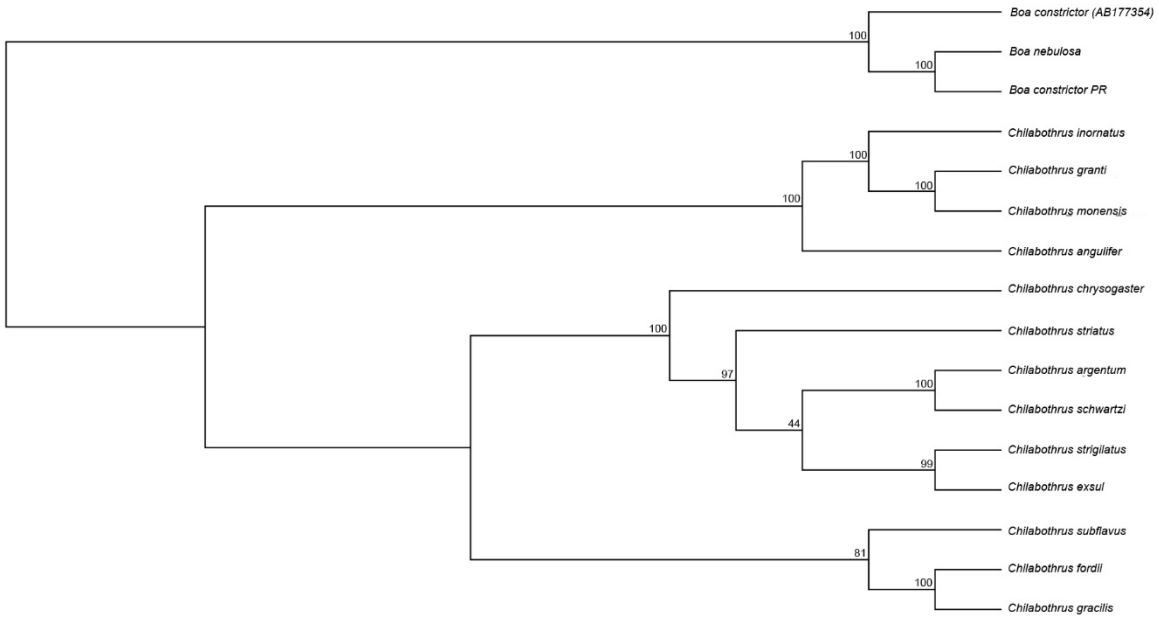

Figure 4. Maximum-likelihood phylogeny of aligned concatenated protein-coding loci.

Figure 4 Maximum-likelihood phylogeny of 13 *Chilabothrus* species and 3 species from the genus *Boa.* Phylogeny was generated using the RAxML algorithm in GENEIOUS. Numbers at nodes represent bootstrap support.

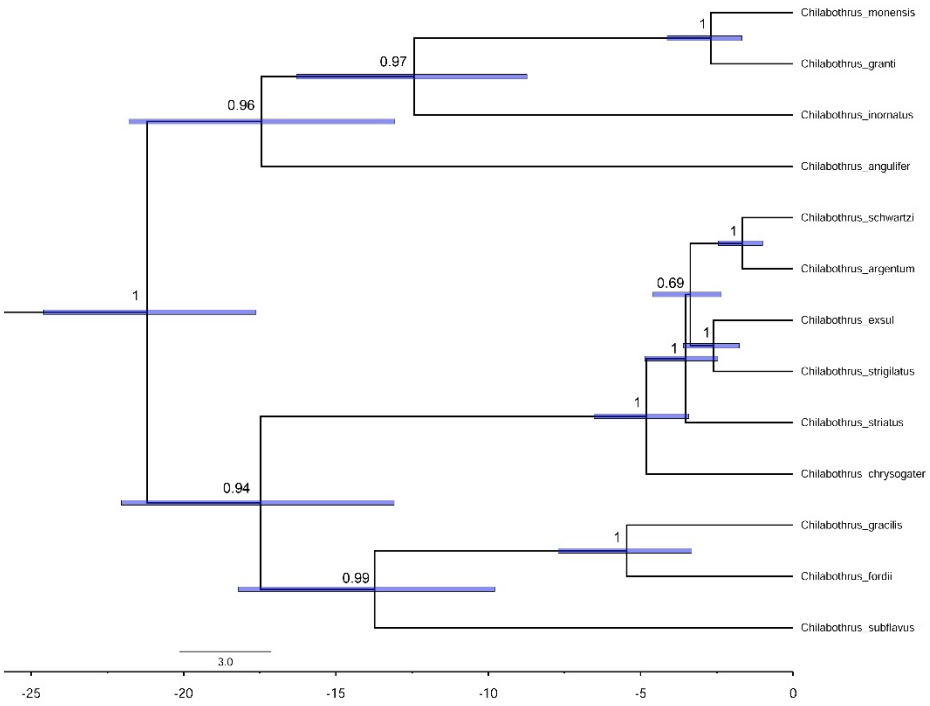

Figure 5. Maximum clade credibility tree.

Figure 5 Time-calibrated coalescent tree of aligned concatenated protein-coding loci for all 13 *Chilabothrus* species. Numbers at nodes represent posterior probabilities times, estimated by constraining the root node of *Chilabothrus* using a normal prior with a mean of 21.7 Mya and a standard deviation of 1.8 Mya. Legend units are million years ago.

4. Discussion

The total lengths (Table 1) of the mitochondrial genomes for the 13 *Chilabothrus* species are comparably shorter than the previously published *Boa constrictor* mitogenome (18,905 bp; GenBank ID: AB177354)[24] used as a reference for alignment. This is because the mitochondrial by-catch data did not obtain all regions of the mitogenome. In particular, I discovered that control regions (CR1 and CR2) were largely excluded from by-catch data, amounting to ~1,500–2,000 bp of sequence. Nevertheless, the by-catch did yield all of the expected protein-coding regions, and lengths of protein-coding loci were similar to the *B. constrictor* genome (11,295 bp total), with a mean length of 10,595 bp and a range of 5,837–11,318 bp (Table 1). The low end of the protein-coding loci range is attributed to *C. gracilis* which had the least mitogenome coverage with a total length of 8,854 bp (Table 1) owing to fewer sequences having been obtained during initial sequencing. This could easily be remedied by re-sequencing this species.

My phylogenetic analyses support most previously published relationships of neotropical boids, only differing in topology in terms of the relationships of *C. exsul*, *C. strigilatus*, and *C. striatus*. My analyses have reinforced the sister relationship between the two newly-described species *C. argentum*[9] and *C. schwartzi*[14]. The topological arrangements presented (Figure 4) are strongly supported and infer that *C. exsul* and *C. strigilatus* are sister lineages, which was previously unknown. Using mitochondrial by-catch to assemble the entire protein-coding loci provides the ability to accurately determine the evolutionary relationships of *Chilabothrus* snakes, as well as other species. Both maximum-likelihood and Bayesian analyses produced the same topology (Figure 4; Figure 5). Unlike previous studies that have used one protein-coding region, the complete protein-coding mitogenomes have given a strongly supported topology for the *Chilabothrus* phylogeny. Since many of these boas face serious conservation concerns, with two listed as endangered and one as critically endangered on the IUCN Red List, being able to easily provide genomic resources is crucial for their conservation. This method of assembling mitogenomes via UCE by-catch is an affordable and easy way to provide genomic resources for these endangered species, as well as give us insight into the evolutionary histories of these snakes.

Using mitogenomes for phylogenetic studies can be useful for clarifying relationships with disagreements between molecular and morphological analyses. Mitochondrial phylogenetic analyses have been able to confirm the sister relationship between *Python* and *Xenopeltis*, which were supported by previous molecular studies, but not by morphological analyses[35]. Sequencing mitogenomes may also be useful for clearing up evolutionary relationships among *Liolaemus* lizards, which is a genus of lizards with recent diversification causing controversy between these species. Studies analyzing mitochondrial genomes have suggested that the derived secondary structures of tRNAs and tandem repeats in the control regions may be useful for phylogenetic analyses of these species[36]. While this type of sequencing for mitochondrial genomes is incredibly useful for phylogenetic or phylogeographic studies, mtDNA evolved much faster than nuclear DNA and may not be entirely accurate for inferring relationships among taxa that speciated a long time ago. There are discrepancies among mitochondrial and nuclear analyses inferring early snake relationships. While multiple mitochondrial analyses have suggested that snakes are sister to the Amphisbaenia/lacertiform clade, nuclear studies have placed snakes with anguimorphs and iguanids[37]. Further research should focus on sequencing missing regions to complete the mitochondrial genomes for these species, as well as generating nuclear DNA. This mitochondrial information, paired with nuclear DNA, will be useful for producing an updated boid phylogeny and is an affordable and useful method for resolving evolutionary relationships among groups of taxa at the genus level and above.

5. Acknowledgments

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