

Aardwolf population diversity and phylogenetic positioning inferred using complete mitochondrial genomes

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The aardwolf (*Proteles cristata*) is a unique, insectivorous species of the family Hyaenidae. It occupies a disjunct range in eastern and southern Africa and is possibly a remaining member of a historical dog-like hyaena clade. Although both local and global population status and trends are largely unknown, aardwolves are not uncommon in arid grasslands and the IUCN conservation status of Least Concern appears to be justified. However, they are one of the least studied of the four hyaena species, and we have scarce information about the genetic structure of aardwolf populations. Here, we present the first complete aardwolf mitochondrial genomes and provide novel information about aardwolf evolutionary origins and genetic structure within a single population. Through the investigation of complete mitochondrial genomes from five individuals from a single population within South Africa, we find the mitochondrial diversity of this population to be neither particularly high nor low compared to a number of other mammalian species. Moreover, we also provide additional evidence towards the basal position of the aardwolf within Hyaenidae with a divergence time of 13.0 Ma (95% CI 10.1–16.4 Ma) from all other extant hyaena species.

Keywords: mitochondria, aardwolf, hyaena, genetic diversity, phylogenetics.

INTRODUCTION

The Hyaenidae, a family within the order Carnivora, is believed to have arisen approximately 25 million years ago (Ma) during the Late Oligocene (Werdelin & Solounias, 1991). This family was once highly diverse and at the peak of its diversity, during the Late Miocene, consisted of more than 80 species across Europe, Asia, Africa and North America (Werdelin & Solounias, 1991). However, in contrast to its past diversity, the family now comprises only four extant morphologically and ecologically distinct genera, each of which consists of only a single species. These extant lineages have been molecularly estimated to have arisen maximally ~15 Ma (Koepfli *et al.*, 2006) and

may therefore be a subclade within the Hyaenidae which consists of many more extinct basal fossil lineages. These genera are made up of the bone cracking *Crocota crocuta* (spotted hyaena), *Hyaena hyaena* (striped hyaena) and *Parahyaena brunnea* (brown hyaena) and, the clear outlier within the extant species, the insectivorous *Proteles cristata* (aardwolf) (Koepfli *et al.*, 2006). In addition to being the only non-bone cracking hyaena species alive today, the aardwolf is morphologically more similar to canids and is believed to be a morphologically primitive hyaena (Werdelin & Solounias, 1991).

The aardwolf has a discontinuous distribution across Africa and occurs in two distinct areas separated by approximately 1500 km: east and northeastern Africa and southern Africa. This distribution pattern is believed to be due to the distribution of their main food source, termites of

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the genus *Trinervitermes* which are entirely absent from the central African woodlands (Coe & Skinner, 1993; Kingdon *et al.*, 2013). Despite a general lack of abundance data, the aardwolf is believed to have stable population numbers and is listed as Least Concern by the International Union for Conservation of Nature (IUCN) (Green, 2015).

Despite previous genetic work looking into the phylogenetic positioning of the aardwolf within the Hyaenidae (Koeplli *et al.*, 2006), to date no complete mitochondrial genome has been published. In addition, there is a general lack of data on its genetic structure and variation, both within and between populations. Such data have been highlighted as a research priority for the southern parts of the species' range (deVries *et al.*, 2016). Here, we present not only a novel aardwolf mitochondrial genome but also a first glimpse into the population structure and the genetic variation of the aardwolf through investigation of the mitochondrial genomes of five individuals from a single population within South Africa. Moreover, using these mitochondrial genomes, we provide additional evidence as to the aardwolf's phylogenetic positioning within the extant Hyaenidae family tree.

MATERIALS AND METHODS

We used skin tissue samples collected from five aardwolf captured for an ecological research project on Benfontein Game Reserve outside of Kimberley, central South Africa (28°52'S, 24°50'E) (Marneweck *et al.*, 2015). The reserve covers approximately 11 400 ha and consists mainly of dry Karoo shrubland, arid grasslands and Kalahari thornveld (Dalerum *et al.*, 2017). The animals were captured from 2008 to 2012 and resided in neighbouring home ranges (Marneweck *et al.*, 2015). Captures were carried out under a permit from the animal care and use committee of the University of Pretoria (EC031-07) and permits from the provincial government in the Northern Cape (FAUNA 846/2009, FAUNA 847/2009). Aardwolves were immobilized by remote injection with 36.0 mg ketamine hydrochloride and 0.6 mg medetomidine hydrochloride using a CO₂-powered dart gun (Kotze *et al.*, 2012). All immobilizations were carried out by personnel registered by the South African Veterinary Council. Genetic samples consisted of a small piece of skin collected at the ear tip. Samples were stored in 95% ethanol at -20°C until analyses.

We extracted DNA from the tissue samples using a Zymo Genomic DNA Clean & Concentra-

tor[®] extraction kit (Zymo Research, U.S.A.), following the manufacturer's protocol. DNA extracts were built into Nextera Illumina sequencing libraries (Inqaba Biotec, Pretoria) and were sequenced on an Illumina Nextseq 500 at the University of Potsdam, Germany, using 2 × 150 bp paired-end reads. We then used Cutadapt v1.4 (Martin, 2011) to trim Illumina adapter sequences from the raw reads and remove reads shorter than 30 bp. Paired end reads with overlapping regions were merged using FLASH v1.2.10 (Magoçê & Salzberg, 2011). As there was no complete aardwolf mitochondrial genome to use as a mapping reference, we first constructed one from a single individual (Aardwolf15). We randomly subsampled 40 million merged reads from Aardwolf15 using seqtk (Li, 2012) and then removed PCR duplicates using Prinseq (Schmieder & Edwards, 2011). The remaining reads were treated as single end and underwent an iterative mapping approach using MITObim v1.8 (Hahn *et al.*, 2013), a wrapper script for the Mira v4.0.2 (Chevreux *et al.*, 1999) assembler following an approach similar to Westbury *et al.* (2017). We implemented MITObim using default parameters apart from mismatch values, where we used values from 0% to 3%. Six independent runs in total were performed using two different extant hyaena reference bait sequences; *Crocuta crocuta* (Genbank accession JF894377.1) and *Hyaena hyaena* (Genbank accession NC_020669.1). Mira output maf files were converted to sam files and visualized using Geneious v9.0.5 (Kearse *et al.*, 2012). We constructed initial mismatch value consensus sequences from all six assemblies using a minimum read coverage of 50x, ignoring gaps and used a 75% base call threshold. We then aligned these six sequences using Mafft v7.271 (Kato & Standley, 2013) and built a final consensus sequence by ignoring gaps and implementing a 75% base call threshold in Geneious. An automatic annotation of this final consensus sequence was performed using MITOS (Bernt *et al.*, 2013) and more precise manual annotations were performed using Geneious.

The constructed mitochondrial genome then served as a reference sequence for subsequent mitochondrial mapping analyses. We mapped the trimmed and merged reads from our remaining four individuals to the reconstructed reference sequence with BWA v0.7.15 (Li & Durbin, 2009), using the mem algorithm and default parameters and parsed the mapped files using Samtools v1.3.1 (Li *et al.*, 2009). The consensus sequences

were constructed utilizing the consensus base call method and only considering reads of mapping quality greater than 25 and bases of phred quality scores greater than 25 with ANGSD v0.913 (Korneliussen *et al.*, 2014).

We then aligned our aardwolf reference mitochondrial genome with a number of mitochondrial genomes from members within Feliformia (Supplementary Table S1) using Mafft. Next, we manually excluded the control region from further analyses due to its high variability that can lead to difficulties in a confident and meaningful alignment between species in this region. We then manually annotated the alignment for tRNAs, rRNAs, and protein coding regions to be used as input for Partitionfinder2 (Lanfear *et al.*, 2017), which was used to group each mitochondrial genomic feature into partitions and then find the optimal substitution model for said partition (Supplementary Table S2). Using this alignment, we constructed a phylogenetic tree using BEAST v1.8.4 (Drummond & Rambaut, 2007) on the Cipres server (Miller *et al.*, 2010) specifying the partitions and substitution models as determined *via* Partitionfinder2, an uncorrelated lognormal relaxed clock model, and a birth-death speciation process (Gernhard, 2008). We further specified five exponentially distributed fossil calibration points (Supplementary Table S3) in the phylogeny based on those used in previous phylogenetic dating analyses (Eizirik *et al.*, 2010; Pajimans *et al.*, 2017). We ran the Markov Chain Monte Carlo (MCMC) chain for 10 million generations, sampling every 10 000 generations, and assessed convergence of the posteriors (ESS > 200) using Tracer v1.6 (Rambaut *et al.*, 2014). We extracted the maximum clade credibility tree with node heights scaled to the median of the posterior sample. Furthermore, in order to investigate the utility of single mitochondrial genes for a dated phylogenetic analysis, we repeated the above approach using the three most intraspecifically variable genes from the aardwolf dataset, COX1, Cytb and NAD3, independently and with all three genes combined as one dataset.

We also aligned our five complete aardwolf mitochondrial genomes using Mafft and constructed a median joining haplotype network using PopART (Leigh & Bryant, 2015). From the same alignment, we calculated the mitochondrial diversity of the aardwolf by calculating the overall mean pairwise distances (k), treating gaps and missing data as complete deletions using MEGA6 (Tamura *et al.*, 2013). This k value was then compared against

previously published values from a number of other mammalian species (Westbury *et al.*, 2018). Furthermore, in order to investigate other regions that may be useful in future phylogeographic studies of the aardwolf, we calculated the total and average number of variable regions in each protein coding gene and the control region. To make results comparable between different regions, we further calculated the average number of variable positions per site within each region (Supplementary Table S4).

RESULTS

We produced five complete aardwolf mitochondrial genomes (Genbank accession codes: MH662441–MH662445) using an initial iterative mapping approach of one individual followed by subsequent mapping of four other individuals to the iteratively mapped individual. The mitochondrial genome of Aardwolf15 contained 17 159 bp, all 13 protein-coding genes with expected open reading frames, 22 transfer RNA genes, and two ribosomal RNA genes found within a typical vertebrate mitochondrial genome. We interpret these results as support for our data representing a correct and complete aardwolf mitochondrial genome. We found support for a basal placement of the aardwolf within the Hyaenidae, with a similar but slightly deeper mean divergence time of 13.0 Ma (CI 10.1–16.4 Ma) compared to previous findings (10.6 Ma, (95% CI 7.3–15.0 Ma, Koepfli *et al.*, 2006). We placed *Proteles* with high confidence (posterior probability of 1) as a sister clade containing the bone crushing hyaena genera (*Parahyaena*, *Hyaena* and *Crocota*) (Fig. 1).

When using the three genes Cytb, COX1, and NAD3, in a single combined phylogenetic analysis, we recovered a divergence time of 12.5 Ma (95% CI 8.9–16 Ma). When using just the COX1 gene, we recovered a divergence time of 10.6 Ma (95% CI 6.6–14.9 Ma). However, even after running the BEAST analysis for 100 million generations, neither of the NAD3 and Cytb datasets reached convergence and therefore, no results were obtained for these genes individually.

The haplotype network analysis showed three different haplotypes, defined as at least one substitution within the complete mitochondrial genome, that were fairly distant from one another (between 31 to 50 substitutions between haplotypes). However, we did find one haplotype to be shared between three individuals, Aardwolf08, 09

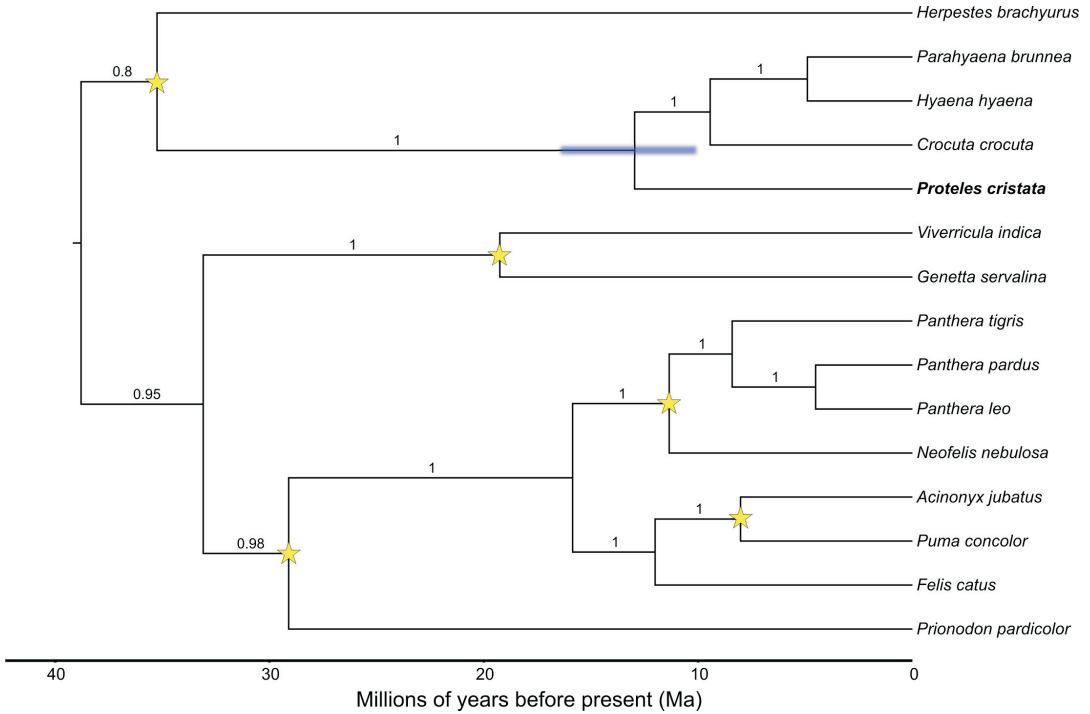


Fig. 1. Molecularly dated Bayesian phylogenetic tree constructed using the complete mitochondrial genomes of all extant Hyaenidae and various other members of the Feliformia suborder. Numbers on branches represent posterior probability support values, stars represent the branch nodes constrained by fossil calibration points and the light purple bar represents the 95% credibility interval for the divergence time of *Proteles* from the other extant hyaena genera. X axis represents time in millions of years.

and 15 (Fig. 2). Finally, we found the mitochondrial diversity (*k*) of this population to be 31 (Fig. 3), placing it in an intermediate position between the other species included in this comparison. This diversity estimate was 7.75 times higher than the lowest in the comparison (the brown hyaena) and 6.12 times lower than the highest in this comparison (the chimpanzee (*Pan troglodytes*)).

We found 17 variable positions within the control region, making it the mitochondrial region with the highest total number of variable positions. The control region also had the highest average number of variable positions per site (0.0182). All protein coding gene regions had between 1 and 9 variable positions and an average number of variable positions of between 0.0010 and 0.0145 per site (Supplementary Table S4).

DISCUSSION

Through the construction of five mitochondrial genomes, we provide a first glimpse into the genetic diversity of the aardwolf. We also provide additional evidence for the phylogenetic positioning of this last remaining dog-like hyaena species.

The mitochondrial genetic diversity was neither high nor low when compared to a number of other

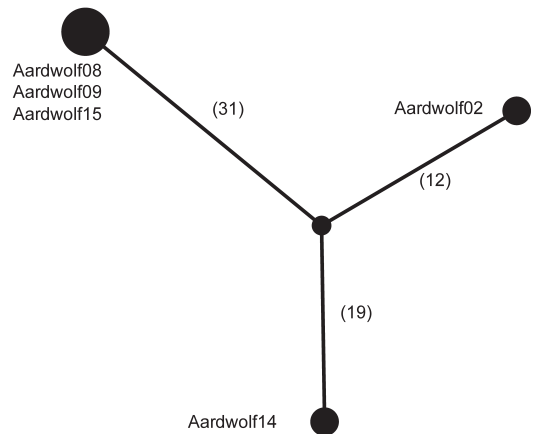


Fig. 2. Median joining haplotype network constructed using the complete mitochondrial genomes of the five aardwolf individuals included in the study. Filled black circles represent individual haplotypes, sizes of the circles represent the number of individuals per haplotype and numbers on the branches represent the number of substitutions between haplotypes.

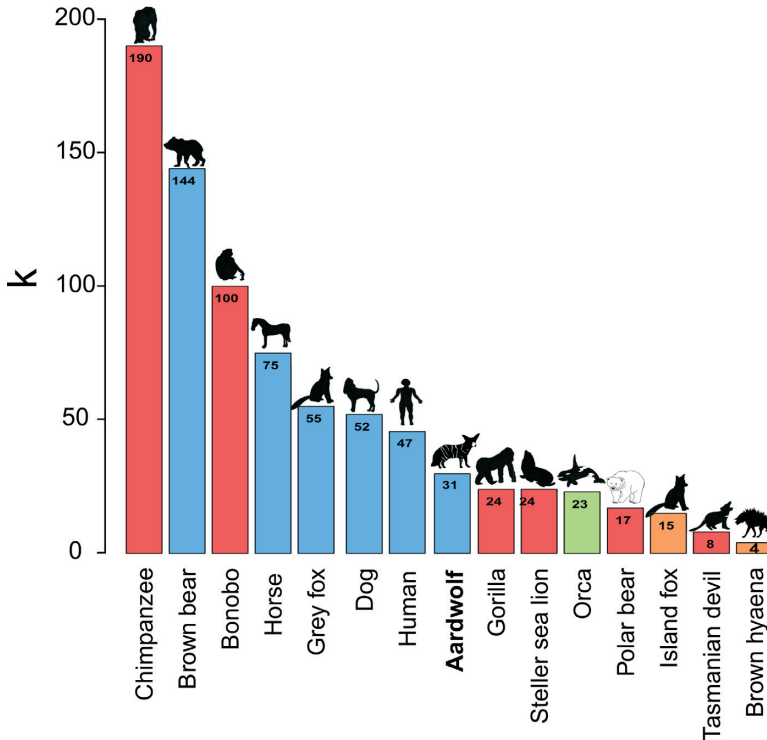


Fig. 3. Mitochondrial diversity comparisons between a number of other mammalian species as seen in Westbury *et al.*, (2018). k represents the average number of substitutions expected between two randomly selected individuals of the same species, bar colours represent conservation status (red, endangered/threatened; orange, near threatened; blue, least concern; green, unavailable) according to the IUCN. Figure modified from Westbury *et al.*, (2018) with permission.

species of varying IUCN conservation levels. This result is unsurprising and is in concordance with the aardwolf's listing of Least Concern by the IUCN (Green, 2015). Furthermore, as this dataset represents only a single population, one would assume the mitochondrial diversity to be higher when considering the species as a whole as new populations should present new haplotypes, especially in the aardwolf since it has a disjunct range (Coe & Skinner, 1993; Kingdon *et al.*, 2013). This assumption is further reinforced by the fact that we found three haplotypes that were relatively distant from one another within a sampling of just five individuals from one location. However, three individuals shared a single mitochondrial haplotype. This result may have arisen by chance in unrelated individuals due to the mitochondrial genome being inherited as a single locus, which can be inherited without any novel substitutions for many generations. However, it could also have arisen due to sampling of maternally related individuals as we sampled individuals with neighbouring home ranges, although we point out that

we have scarce information regarding aardwolf dispersal behaviour.

Interestingly, the aardwolf has a lot more mitochondrial diversity when compared to its family relative, the brown hyaena, which showed a mitochondrial diversity k value of only four across the entire species range as opposed to the aardwolf with a k value of 31 within just a single population. The low genetic diversity in the brown hyaena has been explained by a recent rapid genetic bottleneck (Westbury *et al.*, 2018). However, as the genetic history of the aardwolf is currently unknown, more research, preferably with the help of nuclear genomes, would be needed to better explain the mitochondrial genetic diversity level found within this study. Current results, however, do not suggest the aardwolf has undergone such a bottleneck.

Confident phylogenetic placement of the aardwolf has met with difficulties in the past (Werdelin & Solounias, 1991; Jenks & Werdelin, 1998). Morphologically based phylogenetics were problematic for the aardwolf as, despite retaining many

morphologically primitive characteristics, the aardwolf has a high number of uniquely derived morphological characteristics. One such example is dentition. Dentition is usually a valuable characteristic for morphology based phylogenetic analyses, however, due to the highly reduced dentition of the aardwolf, these comparative analyses were not possible when considering the placement of the aardwolf within the family Hyaenidae (Werdelin & Solounias, 1991). Molecular data, on the other hand, has the ability to resolve this relationship as seen in previous studies (Jenks & Werdelin, 1998; Koepfli *et al.*, 2006). When using complete mitochondrial sequences, we found support for previous findings but with a slightly deeper mean divergence estimate. Furthermore, we found additional support when using subsampled datasets of either three protein coding genes Cytb, COX1, and NAD3 combined or the COX1 gene on its own. This result was not unexpected as the mitochondrial genome is inherited maternally as a single locus so, if enough information is present in the subsampled region, phylogenetic results are expected to be similar. However, in contrast to the COX1 gene alone having sufficient data to reconstruct a molecularly dated phylogeny, the single gene analyses of NAD3 and Cytb datasets did not reach convergence. This could indicate that the data contained in these regions were not sufficient for the current phylogenetic analysis. This could either be due to too few nucleotide positions or, more probably, too few variable positions within these regions between the selected species. Taken together, these results show that even when using a considerably subsampled section (3015 bp (19.4%) or 1538 bp (9.9%)) of the mitochondrial genome (15 533 bp) alignment, robust results can still be obtained. However, it should be noted that as the amount of data in the analysis decreased, the credibility intervals increased, which should also be taken into consideration when selecting markers for future phylogenetic studies.


The convergent results from the current study together with those of previous studies, gives us confidence in both the phylogenetic placement and estimated divergence time of the aardwolf as even when different genetic markers, fossil calibration points and comparative species were used, the same results arose. In addition, we found high levels of variation within the control region, COX1 and NAD3 within just this single population of aardwolf, indicating that these regions may

provide sufficient information to uncover phylogeographic structure within the aardwolf even when complete mitochondrial genomes are not available.

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Supplementary material to:

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Supplementary information

Supplementary Table S1: Species and the accession codes for the complete mitochondrial genome sequences used in the phylogenetic analysis.

Genus	Species	Genbank accession code
<i>Parahyaena</i>	<i>brunnea</i>	MF593941
<i>Crocuta</i>	<i>crocuta</i>	F894377.1
<i>Hyaena</i>	<i>hyaena</i>	NC_020669.1
<i>Herpestes</i>	<i>brachyurus</i>	KY117547.1
<i>Acinonyx</i>	<i>jubatus</i>	AY463959.1
<i>Felis</i>	<i>catus</i>	U20753.1
<i>Genetta</i>	<i>servalina</i>	KX891744.1
<i>Panthera</i>	<i>pardus</i>	KP001507.1
<i>Neofelis</i>	<i>nebulosa</i>	DQ257669.1
<i>Panthera</i>	<i>leo</i>	KP001506.1
<i>Panthera</i>	<i>tigris</i>	EF551003.1
<i>Prionodon</i>	<i>pardicolor</i>	KX447638.1
<i>Puma</i>	<i>concolor</i>	KX808231.1
<i>Viverricula</i>	<i>indica</i>	NC_025296.1

Supplementary Table S2: Fossil calibration constraints used in the dated phylogenetic analysis.

Calibration point	Hard minimum age	Soft maximum age (95% of distribution)
Herpestes/Hyaenidae split	16.4	50
Genetta/Viverricula split	11.2	50
Prionodon/Felidae split	28	50
Neofelis/Panthera split	3.8	16
Puma/Acinonyx split	3.8	10

Supplementary Table S3: Partition name, genomic features, and substitution model for each partition implemented in the dated phylogenetic analysis.

Partition name	Substitution model	Features in partition
p1	HKY+I+G	tRNA-Met, tRNA-Asn, tRNA-Pro, tRNA-Phe, tRNA-Thr, 16S, 12S, tRNA-Cys, tRNA-Tyr, tRNA-Leu2, tRNA-Leu, tRNA-Val, tRNA-Ile, tRNA-Arg, tRNA-Glu, tRNA-Ala, tRNA-Ser2
p2	HKY+I+G	ND1, Cytb, ND5, ND3, ND4, ND6, ATP8
p3	HKY+G	tRNA-Gly, tRNA-Gln, tRNA-His, tRNA-Asp, tRNA-Lys
p4	HKY+I+G	ND2, tRNA-Trp
p5	HKY+I+G	tRNA-Ser, ATP6, COX1, COX2, ND4L, COX3

Supplementary Table S4: Nucleotide variability found within protein coding genes and the control region calculated from our sampling of aardwolf mitochondrial genomes.

Gene	Number of variable positions	Number of basepairs(bp)	Ratio of variable positions/total bp
Control region	17	933	0.0182
COXI	9	1538	0.0059
COXII	1	680	0.0015
COXIII	3	782	0.0038
Cytb	4	1133	0.0035
NAD1	3	935	0.0032
NAD2	1	1028	0.0010
NAD3	5	344	0.0145
NAD4	4	1367	0.0029
NAD5	4	1805	0.0022
NAD6	2	518	0.0039
ATP6	4	674	0.0059
ATP8	1	194	0.0052