Journal of Natural History

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/tnah20

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To cite this article: Isabel Tiller, Jayra Penaloza & J. Antonio Baeza (2024) The complete mitochondrial genome of the leopard shark *Triakis semifasciata* (Triakidae), Journal of Natural History, 58:33-36, 1307-1319, DOI: 10.1080/00222933.2024.2350106

To link to this article: https://doi.org/10.1080/00222933.2024.2350106

JOURNAL OF NATURAL HISTORY
2024, VOL. 58, NOS. 33–36, 1307–1319
https://doi.org/10.1080/00222933.2024.2350106

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The complete mitochondrial genome of the leopard shark *Triakis semifasciata* (Triakidae)

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ABSTRACT

The leopard shark *Triakis semifasciata* (family Triakidae) is threatened by habitat loss and targeted by recreational and commercial fishermen in the Eastern Pacific Ocean, from Oregon, USA, to the Gulf of California, Mexico. Despite environmental issues, there are few genetic and genomic resources available for this species. This study assembled the complete mitochondrial genome of *T. semifasciata* from a skin metagenomic sample and described it in detail. The phylogenetic position of *T. semifasciata* amongst closely related species was also examined using mitochondrial protein-coding genes (PCGs). The mitochondrial genome of *T. semifasciata* is 16,613 bp in length and consists of 13 PCGs, two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes. A 30 bp long region was identified as the origin of replication for the light strand (O_L) between the *trnN* and *trnC* genes, and a 974 bp long putative control region (CR) contains the origin of replication for the heavy strand (O_H). The gene order in *T. semifasciata* is identical to that of cofamilial species. An analysis of Ka/Ks ratios for all PCGs yielded values < 1, indicating that all PCGs experience strong purifying selection. All tRNAs exhibit a canonical 'cloverleaf' secondary structure except for *tRNA-Ser1* which lacks the stem of the dihydrouridine (DHU) arm and in place possesses a simple loop. A phylomitogenomic maximum likelihood (ML) analysis did not support the monophyly of the family Trakidae and placed *T. semifasciata* in a sister position to *Hemitriakis japanica*. However, the aforementioned sister position was poorly supported by ML bootstrap values. This study represents a new genomic resource for this commercially and recreationally important species and confirms that mitochondrial genomes can be assembled from skin metagenomic samples as shown before in an unrelated shark species.

ARTICLE HISTORY
Received 17 April 2023
Accepted 24 April 2024

KEYWORDS
Ornamental industry; elasmobranch; mitogenome; conservation biology

Introduction

Carcharhiniformes is the largest order of sharks with 228 extant recognised species, representing more than half of living sharks (Cappetta 1987). This clade exhibits an impressive disparity in terms of morphology, behaviour, physiology, and ecology

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/00222933.2024.2350106.

Published online 03 Sep 2024

(Compagno 2008). Among them, the leopard shark *Triakis semifasciata* (family Triakidae) is an important target species for recreational fishermen, small-scale commercial fisheries, and marine aquaria collectors (Cailliet 1992). *Triakis semifasciata* inhabits bays and estuaries in the Eastern Pacific Ocean, from Oregon, USA, to the Gulf of California in Mexico (Smith and Abramson 1990; Ebert and Ebert 2005). The leopard shark lives in water less than 4 m deep with sandy or muddy bottoms and is dependent on estuaries for breeding, nursing, and foraging (Ebert 2003; Carlisle and Starr 2009). Leopard sharks are robust and active swimmers that form large and nomadic schools, segregated by size and gender, but congregate or travel with other elasmobranchs (Carlisle *et al*. 2015), including bat rays *Myliobatis californica* and smoothhound sharks *Mustelus mustelus* (Hull *et al*. 2018). *Triakis semifasciata* feeds largely on teleost fish, shrimps, clams, crabs, and echiuroid worms (Cailliet 1992) and experiences predation from larger sharks, including the white shark *Carcharodon carcharias* and sevengill shark *Notorynchus cepedianus* (Smith 2007).

The life history of *T. semifasciata* is well documented (Ackerman 1971; Smith and Abramson 1990; Cailliet 1992; Kusher *et al*. 1992; Ebert 2003; Lopez *et al*. 2006; Lewallen *et al*. 2007). *Triakis semifasciata* is a long-lived, late maturing, and low-fecundity species (Cailliet 1992; Kusher *et al*. 1992). Females typically reach sexual maturity between 10 and 15 years and males between 7 and 13 years (Kusher *et al*. 1992). As an aplacental viviparous species, young hatch inside the uterus and are nourished by yolk for 10–12 months before parturition (López *et al*. 2006). *Triakis semifasciata* has a low fecundity of 7–36 offspring per female per reproductive season (Ackerman 1971). Females and males aggregate during the breeding season but form segregated groups outside of breeding season (Ebert 2003). Some individuals return to the same aggregation sites annually (Nosal *et al*. 2014), though large-scale movements appear to be limited (Lewallen *et al*. 2007). All of these life history features suggest that *T. semifasciata* is susceptible to overexploitation (Kusher *et al*. 1992).

In past decades, harvesting rates of the leopard shark has increased (Smith and Abramson 1990), as its meat is frequently sold in California fish markets for its 'excellent quality' (Ebert 2003). Furthermore, pups are commonly poached for the cold-water aquarium trade (Carlisle *et al*. 2015). Other than overfishing and poaching, habitat loss is among the greatest current threats to *T. semifasciata*. Over 90% of estuarine wetlands in California were altered or lost in the last century (Dahl 1990), decreasing the amount of and accessibility to vital breeding and nursery habitat. *Triakis semifasciata* is listed as of 'Least Concern' by the International Union for Conservation of Nature and is currently managed under the Pacific Fishery Management Council's Groundfish Fishery Management Plan (Carlisle *et al*. 2015).

Despite conservation issues, little genetic and genomic information exists for *T. semifasciata*. Current genetic evidence suggests the leopard shark exhibits significant population structure (Barker *et al*. 2015): northern and southern California populations are distinct, and populations from Mexico are genetically isolated from those in California (Lewallen *et al*. 2007; Barker *et al*. 2015). The current status of population trends are unknown (Carlisle *et al*. 2015).

This study aimed, for the first time, to assemble and characterise the mitochondrial genome of the leopard shark *T. semifasciata*. We followed the recommendations in Baeza (2022) to conduct a detailed analysis of the newly assembled mitochondrial genome: we estimated nucleotide composition and the secondary structure of tRNAs, and conducted

selective pressure and codon usage analyses on the PCGs. Additionally, a detailed characterisation of the putative control region was conducted. Lastly, we examined the phylogenetic position of *T. semifasciata* in the family Triakidae using translated mitochondrial PCGs. This complete and detailed characterisation of the mitochondrial genome is a critical step to support conservation measures for *T. semifasciata*.

Methods

DNA genomic dataset

The raw sequence dataset used to assemble the mitochondrial genome of *T. semifasciata* was generated by Doane *et al*. (2022) and employed by these authors to describe the skin microbiome. A total of 1,713,261 paired-end (PE) reads were retrieved from NCBI's GenBank (SRA accession number SRR17931172) and detailed information on sampling, DNA extraction, and sequencing methods can be found in the aforementioned study. The totality of the reads available in GenBank was used for mitochondrial genome assembly.

Mitochondrial genome assembly

Assembly of the mitochondrial genome from the skin metagenome sample was conducted using the target-restricted-assembly pipeline GetOrganelle v. 1.2.3 (Jin *et al*. 2020). GetOrganelle uses a seed-and-extend algorithm that assemble organelles, including mitochondrial genomes, from whole genome sequencing (WGS), including metagenomic datasets, starting from a related or distant single 'seed' sequence (Jin *et al*. 2020). The complete mitochondrial genome of the cofamilial shark *Mustelus mustelus* (NC_039629) retrieved from GenBank was used as the 'seed'. The run used k-mer sizes of 21, 55, 85, and 115 and reads were not quality trimmed prior to assembly using the GetOrganelle pipeline, following the developer's guidelines (Jin *et al*. 2020). The software Bandage (Wick *et al*. 2015) was used to visualise the assembly graph generated by GetOrganelle. We predicted that a circularised sequence ~16,500–17,000 bp in length would be observed among the contigs if GetOrganelle successfully assembled the mitochondrial genome of *T. semifasciata*.

Annotation and analysis of the assembled mitochondrial genome

The assembled mitochondrial genome of *T. semifasciata* was annotated using the web servers MITOS (http://mitos.bioinf.uni-leipzig.de – Bernt *et al*. 2013) and MITOS 2 (http://mitos2.bioinf.uni-leipzig.de – Bernt *et al*. 2013) with the vertebrate genetic code. Corrections to the start and stop codons were conducted using the web server ExPASy (http://web.expasy.org). The nucleotide composition of the entire mitochondrial genome was estimated in the software MEGA 11 (Kumar *et al*. 2021). The entire mitochondrial genome was visualised as a circular map using the web server GenomeVx (http://wolfe.ucd.ie/GenomeVx/ – Conant and Wolfe 2008).

Relative synonymous codon usage (RSCU) of the PCGs was analysed using the program EZcodon in the web server EZmito (http://ezmito.unisi.it/ezcodon – Cucini *et al*. 2021). Selective pressures on the mitochondrial PCGs were examined by estimating rates of

nonsynonymous and synonymous substitutions. Ka (number of nonsynonymous substitutions per nonsynonymous site), Ks (number of synonymous substitutions per synonymous site), and the Ka/Ks ratio were calculated for each PCG using the software Ka_Ks_calculator2 (Wang *et al*. 2010). The aforementioned values were estimated using the cofamilial *M. griseus* (NC_023527) as an outgroup. The analyses were conducted using the γ-MYN model as it accounts for variable mutation rates across different sites within each PCG. If the Ka/Ks ratio was greater than 1, equal to 1, or less than 1, genes were predicted to experience diversifying selection, neutral selection, or purifying selection, respectively (Wang *et al*. 2010).

tRNA genes were identified using the software MiTFi (Juhling *et al*. 2012) as implemented in MITOS2, and their secondary structures were visualised in the web server Forna (http://rna.tbi.univie.ac.at/fornia/ – Kerpedijev *et al*. 2015).

The control region (CR) in the mitochondrial genome of *T. semifasciata* was analysed in detail. Repeats within this long non-coding region were found using the web servers Tandem Repeat Finder (https://tandem.bu.edu/trf/trf.basic.submit.html – Benson 1999) and Microsatellite Repeats Finder (http://insilico.ehu.es/mini_tools/microsatellites/ – Bikandi *et al*. 2004). The secondary structure of the CR was predicted in the web server RNA-Structure (https://rna.urmc.rochester.edu/RNAstructureWeb – Bellaousov *et al*. 2013).

Phylogenetic position of Triakis semifasciata

We explored the phylogenetic position of the leopard shark in the family Triakidae based on translated PCGs. For this purpose, we used the newly assembled mitochondrial genome of *T. semifasciata* plus other mitochondrial genomes belonging to five cofamilial species available in the GenBank database (consulted 10 April 2023). Mitochondrial genomes belonging to 14 other shark species from closely related families (eg Carcharhinidae) were used as outgroups.

For the phylogenetic analysis, amino acid residues from each of the 13 PCGs were first aligned using the program Clustal Omega (Sievers and Higgins 2014); poorly aligned regions were removed with trimAl (Capella-Gutiérrez *et al*. 2009), and the best partition and fitting models of sequence evolution for the dataset were selected using ProtTest (Abascal *et al*. 2005). Lastly, the concatenated and partitioned PCG amino acid alignments were used to conduct a maximum likelihood (ML) phylogenetic tree analysis using the program IQ-TREE (Nguyen *et al*. 2015). The robustness of the ML tree topology was determined by executing 1000 bootstrap pseudo-replicates of the tree search.

Results and discussion

The pipeline GetOrganelle assembled and circularised the mitochondrial genome of the leopard shark *T. semifasciata* (NCBI's GenBank accession number: OQ612633) with a coverage equal to 34x. The complete mitochondrial genome of *T. semifasciata* was 16,613 bp long and contained 13 PCGs, two ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes (Table 1, Figure 1), and a CR 974 bp long. The gene order observed in *T. semifasciata* is identical to that reported for cofamilial sharks, including, among others, *Mustelus griseus* (Chen *et al*. 2016) and *Hemitriakis japanica* (Wang *et al*. 2022).

Table 1. Mitochondrial genome of *Triakis semifasciata*: arrangement and annotation.

Name	Type	Start	Stop	Strand	Length (bp)	Start	Stop	Anticodon	Continuity
trnF(gaa)		1	69	(+)	69				1
rrnS		71	1023	(+)	953				−3
trnV(tac)		1021	1092	(+)	72				23
rrnL		1116	2763	(+)	1648				−1
trnL2(taa)		2763	2837	(+)	75				0
nad1		2838	3812	(+)	975	ATG	TAA		0
trnI(gat)		3813	3882	(+)	70				1
trnQ(ttg)		3884	3955	(-)	72				0
trnM(cat)		3956	4024	(+)	69				0
nad2		4025	5071	(+)	1047	ATG	TAG		−2
trnW(tca)		5070	5140	(+)	71				1
trnN(tgc)		5142	5210	(-)	69				0
trnN(gtt)		5211	5283	(-)	73				6
OL		5290	5319	(+)	30				1
trnC(gca)		5321	5389	(-)	69				1
trnY(gta)		5391	5460	(-)	70				1
cox1		5462	7018	(+)	1557	GTG	TAA		0
trnS2(tga)		7019	7089	(-)	71				3
trnD(gtc)		7093	7162	(+)	70				7
cox2		7170	7860	(+)	691	ATG	T(AA)		0
trnK(ttt)		7861	7934	(+)	74				1
atp8		7936	8103	(+)	168	ATG	TAA		−10
atp6		8094	8777	(+)	684	ATG	TAA		−1
cox3		8777	9562	(+)	786	ATG	TAA		2
trnG(tcc)		9565	9634	(+)	70				0
nad3		9635	9985	(+)	351	ATG	TAG		−2
trnR(tcg)		9984	10,053	(+)	70				0
nad4l		10,054	10,350	(+)	297	ATG	TAA		−7
nad4		10,344	11,724	(+)	1381	ATG	T(AA)		0
trnH(gtg)		11,725	11,793	(+)	69				0
trnS1(gct)		11,794	11,860	(+)	67				0
trnL1(tag)		11,861	11,932	(+)	72				0
nad5		11,933	13,762	(+)	1830	ATG	TAA		−5
nad6		13,758	14,279	(-)	522	ATG	AGG		0
trnE(ttc)		14,280	14,349	(-)	70				2
cob		14,352	15,497	(+)	1146	ATG	TAG		−1
trnT(tgt)		15,497	15,568	(+)	72				2
trnP(tgg)		15,571	15,639	(-)	69				278
CR		15,640	16,613		974				
OH		15,918	16,612	(+)	695				

The overall nucleotide composition on the mitochondrial genome's positive DNA strand is T = 30.6%, C = 24.8%, A = 30.5%, and G = 14% (Supplementary material, Table S1) with an AT content equal to 61.1%. In cofamilial species, AT content is similar to that reported here for *T. semifasciata* and ranges from a minimum of 60% in *H. japanica* (Wang *et al*. 2022) to a maximum of 61.8% in *Mustelus manazo* (Cao *et al*. 1998). The disproportional use of nucleotides in the mitochondrial genome of *T. semifasciata* and related species may be a result of environmental selective pressures on codon usage (Hildebrand *et al*. 2010).

In the mitochondrial genome of *T. semifasciata*, the most frequently used start codon was ATG (12 out of 13 PCGs used it). The least frequently used start codon was GTG (one PCG used it: *cox1*). Identical start codon usage in PCGs occurs in *M. griseus* (Chen *et al*. 2016) and *H. japanica* (Wang *et al*. 2022). The most frequently used stop codon was TAA (seven PCGs used it: Table 1). Three PCGs (*nad2, nad3, cob*) used TAG as a stop codon. Two PCGs (*nad4, cox2*) used incomplete T stop codons (Table 1), similar to that reported before

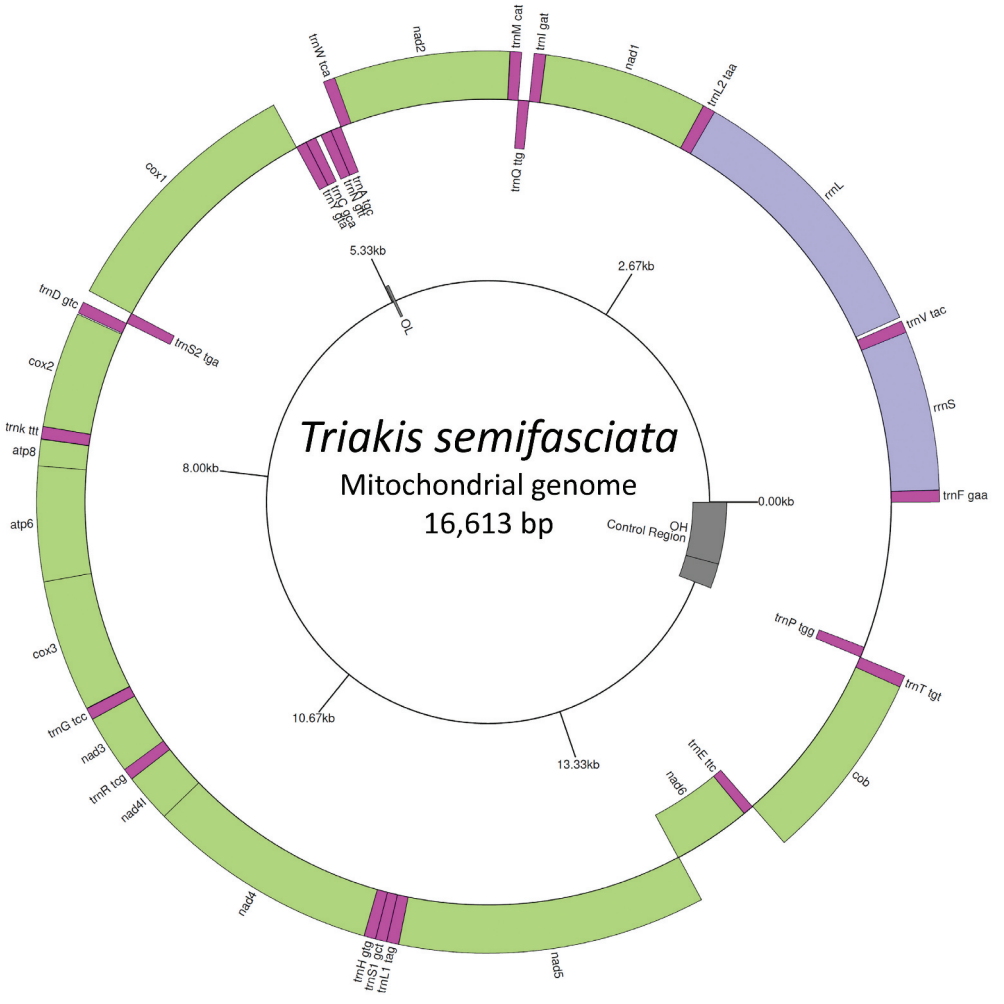

Figure 1. Circular map of the mitochondrial genome of the leopard shark *Triakis semifasciata*.

for *M. griseus* (Chen *et al*. 2016) and *H. japanica* (Wang *et al*. 2022). The least frequent stop codon, used by one PCG (*nad6*), was the AGG end codon, as also reported in *M. griseus* (Chen *et al*. 2016). The most frequently used codons in the PCGs of *T. semifasciata*'s mitogenome are ATT (Ile, N = 220 times used, 5.77% of total), TTA (Leu, N = 205 times used, 5.37% of total), CTA (Leu, N = 155, 4.06% of total), CTT (Leu, N = 143, 3.75% of total), and ATA (Met, N = 139, 3.64% of total). Excluding stop codons, the least frequently used codons were CCG (Pro, N = 2, 0.052% of total), TAG (End, N = 3, 0.07% of total), TCG (Ser, N = 4, 0.10% of total), and AAG (Lys, N = 4, 0.10% of total) (Figure 2). The RSCU values estimated for 38 out of 59 codons used by the mitochondrial PCGs were less than 1.0 and thus exhibited negative codon usage (were not preferred). In turn, 31 codons had RSCU values greater than 1.0 and thus exhibited positive usage biases (Figure 2). Most of the preferred codons were AT-rich, specifically at the third codon position. A similar codon usage profile has been reported before in the cofamilial *H. japanica* (Wang *et al*. 2022) and the more distantly related *Cephaloscyllium umbratile* (family Scyliorhinidae) (Zhu *et al*.

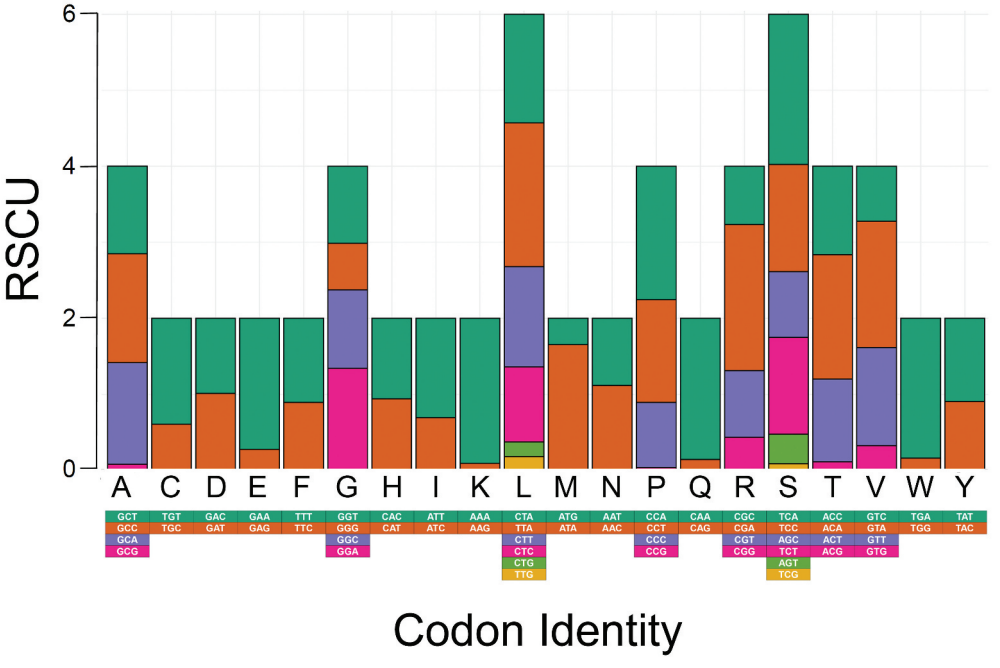

Figure 2. Codon usage in 13 protein-coding genes encoded in the mitochondrial genome of the leopard shark *Triakis semifasciata*.

2017). Most of the codons used by the mitochondrial PCGs of *T. semifasciata* are AT-rich, which is in line with the observed overrepresentation of the same nucleotides in the mitogenomes of *H. japanica* and *C. umbratile* (Zhu *et al*. 2017; Wang *et al*. 2022).

The Ka/Ks ratio calculated for all 13 mitochondrial PCGs in *T. semifasciata* exhibited values < 1 (p value < 0.05 in all cases), indicating these genes have been exposed to purifying (negative) selection, with some genes undergoing more intense selective pressure than others (Figure 3). Specifically, the *nad3* and *nad6* Ka/Ks ratios were 0.153 and 0.126, respectively, which are considerably larger than the Ka/Ks values for the remaining 11 PCGs. Thus, purifying selection appears to be weaker on *nad3* and *nad6* than on other PCGs. The Ka/Ks ratio in *cox1* is 0.0039 which is comparatively lower than that of the remaining genes, suggesting that there is a stronger purifying selection in this gene. Selective pressure analyses have rarely been conducted in sharks, and none have been conducted in other species belonging to the family Triakidae. However, a strong pattern of negative purifying selection has been reported for all mitochondrial PCGs in fishes, including a few studied sharks in the order Carcharhiniformes (the Japanese swellshark *C. umbratile* – Zhu *et al*. 2017).

In the mitochondrial genome of *T. semifasciata*, 21 out of 22 tRNA genes exhibit a canonical 'cloverleaf' secondary structure (Figure 4). In turn, *tRNA-Ser1* lacks the dihydrouridine (DHU) stem as predicted by MiTFi, similar to that reported before for the same tRNA gene in other members of the same order, including *C. umbratile* (Zhu *et al*. 2017). However, the cofamilial sharks *H. japanica* and *M. griseus* exhibited no truncation of *tRNA-Ser1*. Furthermore, *M. griseus* was observed to have a truncated *tRNA-Ser2* (Chen *et al*.

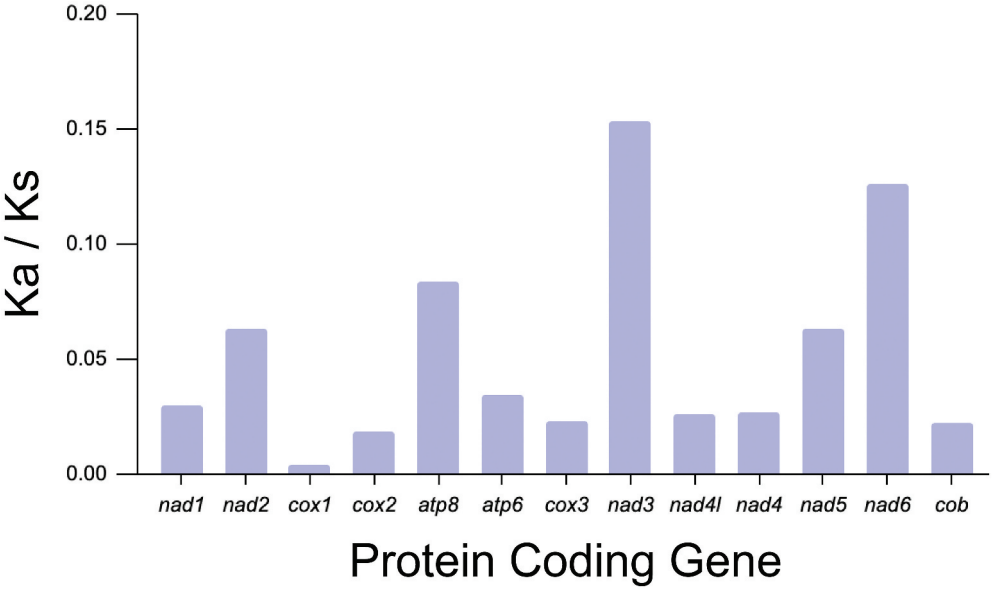

Figure 3. Selective pressure analysis in the mitochondrial protein-coding genes of the leopard shark *Triakis semifasciata*. The estimated Ka/Ks ratio for each protein-coding gene is shown.

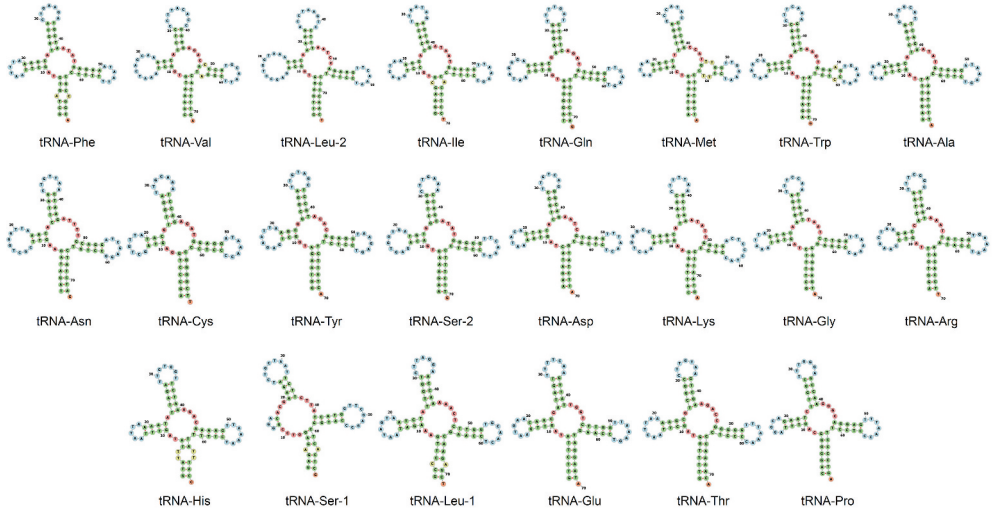

Figure 4. Visualisation of the tRNA secondary structure encoded in the mitochondrial genome of the leopard shark *Triakis semifasciata.*

2016; Wang *et al*. 2022). Despite these findings in *H. japanica* and *M. griseus*, truncation of the tRNA-*Ser1* (loss of the loop, the stem, or the entire D-arm) is commonly observed across eumetazoans, including vertebrates and other shark species (Watanabe *et al*. 2014). It is not clear whether truncated tRNA genes are functional. However, aminoacylation and Ef-TU binding of D arm lacking tRNAs has been demonstrated before in other species (Suematsu *et al*. 2005) and are suggested to aid in the functionality of truncated tRNAs

(Watanabe *et al*. 2014). However, the possibility that truncated tRNAs are non-functional pseudogenes should be explored in the studied species and other elasmobranchs.

In the mitochondrial genome of *T. semifasciata*, the 974 bp long CR is located between *tRNA-Pro* and *tRNA-Phe*, identical to that reported in other cofamilial species (eg *M. griseus* – Chen *et al*. 2016; *H. japanica* – Wang *et al*. 2022). The control region is AT-rich, with an overall nucleotide composition of T = 34.5%, C = 20%, A = 31.5%, and G = 14%. The origin of replication for the heavy strand (O_H) is located within the CR and is 695 bp long. The 30 bp long origin of replication for the light strand (O_L) is not located in the CR but between the *tRNA-Asn* and *tRNA – Cys* genes. The studied CR is shorter than that described for closely related species (eg 1068 bp in *M. manazo* – Cao *et al*. 1998; 1119 bp in *M. griseus* – Chen *et al*. 2016). Overall, the mitochondrial CR of elasmobranchs is known to exhibit extensive variability in length (Kitamura *et al*. 1996; Castro *et al*. 2007; Ramirez-Macias *et al*. 2007; Kousteni *et al*. 2021). In the leopard shark, the mitochondrial CR contains a 46 bp long tandem repeat 5′-(ATA CTA TGC TTA ATC CGC ATT AAT CGA CAT TCC CCT ATA TCA TTA C)-3′ repeated 2.2 times. No tandem repeats have been reported before in the CR of cofamilial species, but tandem repeats have been observed in the blue shark *Prionace glauca* (Chen *et al*. 2015) and the spiny butterfly ray *Gymnura altavela* (Kousteni *et al*. 2021). The CR of *T. semifasciata* contains 16 microsatellites, where most are AA and TT dinucleotide repeats between 3 and 4 times (Supplementary material, Table S1). Finally, the RNA-Structure web server predicted two possible secondary structures and each contained variable numbers and sizes of stem-loops throughout the entire CR sequence (Supplementary material, Figure S1). We argue in favour of additional studies focusing on the characterisation of the CR in sharks and allies to understand the role of this region in mitochondrial genome replication and transcription (Bernt *et al*. 2013).

Our 'total evidence' phylomitogenomic analysis was based on a total of 3800 amino acids, out of which 527 characters were parsimony informative, for a total of six ingroup terminals (representatives of the family Triakidae, including *T. semifasciata*) and 14 outgroup terminals. In the ML phylomitogenomic analysis, all specimens belonging to the family Triakidae clustered together into a single clade; however, bootstrap support (bv) for the monophyletic status of this family was low (bv = 50) (Figure 5). In the poorly supported family Triakidae, our analysis indicated reciprocal monophyly between another poorly supported clade comprising *Triakis semifasciata* and *Hemitrakis japanica* (bv = 50) and a fully supported clade (bv = 100) composed of all representatives of the genus *Mustelus* used in this study. A larger sample of representatives from the family Triakidae is needed to explore phylogenetic relationships in this clade as well as the evolution of reproductive modes (López *et al*. 2006).

Conclusions

For the first time, this study assembled the complete mitochondrial genome of the leopard shark, *T. semifasciata*. This shark species is threatened by habitat loss as a result of environmental degradation from both increased urban runoff and extreme weather events such as floods. Habitat loss is of rising concern as females rely on bays and estuaries for reproduction; a critical nursery site for the species includes the San Francisco Estuary, which has experienced a leopard shark decline over the past couple of years. The comprehensive analysis of the mitochondrial

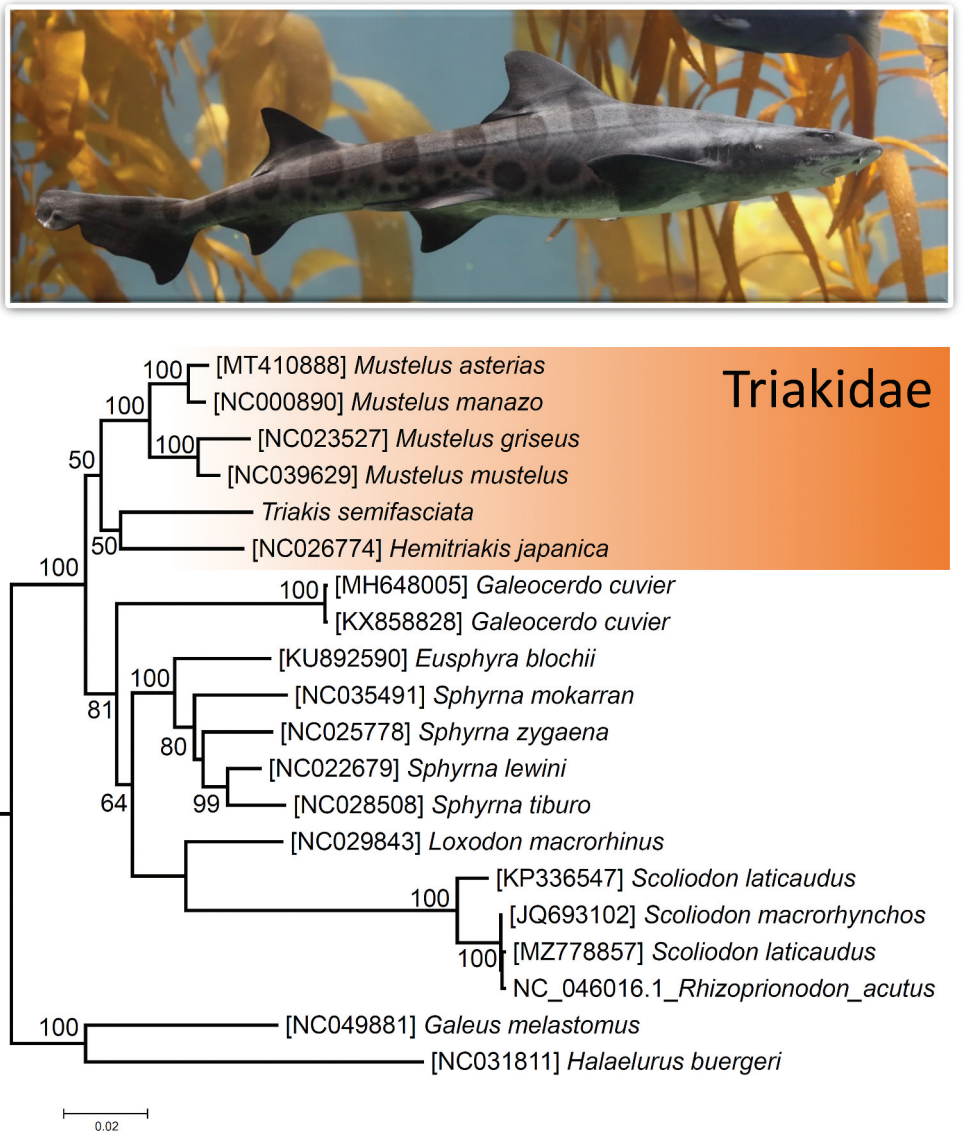

Figure 5. Phylomitogenomic analysis of the leopard shark *Triakis semifasciata* and related species in the family Triakidae. Total-evidence phylogenetic tree obtained from a maximum likelihood analysis based on a concatenated alignment of the 13 protein-coding genes (translated) encoded in the mitochondrial genome. Numbers above or below branches are bootstrap support values for the different internal nodes. Photograph of *Triakis semifasciata* from Matthew Field (used with permisssion).

genome will contribute to biomonitoring and bioprospecting this species using environmental DNA (eDNA). This study will help boost conservation efforts of this and closely related sharks in the poorly studied family Triakidae. This study also demonstrates that mitochondrial genomes can be assembled from skin metagenomic datasets (as in Doane *et al*. 2018) and opens the opportunity to include

maternal genotype as a covariate in studies attempting to understand the conditions explaining the diversity and function of microbiomes in sharks and beyond.

Acknowledgements

J. Antonio Baeza thanks Dr. Vincent P. Richards for bioinformatics support. This project was supported by the Clemson University Creative Inquiry program.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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