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Mitochondrial recovery from shotgun metagenome sequencing enabling phylogenetic analysis of the common thresher shark (*Alopias vulpinus*)

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ABSTRACT

The common thresher shark (*Alopias vulpinus*) is an important fisheries species in the northeastern Pacific Ocean along California, USA and Mexico; yet genetic understanding of this species is incomplete. Using IonTorrent PGM generated metagenomic libraries constructed from the skin surface, we recovered the complete mitogenome of the common thresher shark. The length of the mitogenome was 16,712 basepairs and consisted of 22 tRNA, two rRNA, 13 protein coding sequences, a replication origin and a control region, similar to other *Alopias* spp. The median coverage across the mitogenome was 21 ×, ranging from 1 to 48 × coverage. The mean GC content of the mitogenome was 37.83%. Using the 13 protein coding genes of the mitogenome, we show phylogenetic placement of the common thresher shark with the pelagic thresher (*Alopias pelagicus*; *a posterior* probability of 1). In addition, the inclusion of this mitogenome increased certainty for the placement of the Lamniformes family with the Carcharhiniformes family (*a posterior* probability of 1), rather than with the Orectolobeformes family, as has been previously reported. The availability of the common thresher shark mitogenome will aid phylogenetic inference and population based studies of this important fisheries species.

1. Introduction

The shark genus *Alopias* is comprised of three species: common thresher (*A. vulpinus*), pelagic thresher (*A. pelagicus*), and bigeye thresher (*A. superciliosus*). The common thresher shark (from here on referred to as thresher, unless otherwise noted) is a large-bodied pelagic fish reaching lengths of nearly 500 cm total length (TL) for males and over 600 cm TL for females (Cailliet et al., 1983). These animals are long-lived reaching a maximum age of up to 38 years in the Atlantic Ocean (Natanson et al., 2015). Threshers exhibit low fecundity with an age of maturity that ranges between 8 years for males and 13 years for females (Natanson et al., 2015) and a gestation period of nine months (Goldman, 2005). In addition, threshers have relatively small litter sizes with two to four pups per litter (Cailliet and Bedford, 1983). These slow life history traits result in thresher sharks being susceptible to fishery impacts (Chapple and Botsford, 2013).

Thresher sharks display a circum-global distribution (Compagno, 2001), but primarily live within 40 miles of the coast (Holts, 1988; Litvinov, 1989; Smith et al., 2008). Like their congeners, *A. vulpinus* individuals spend a majority of their time in the epipelagic zones of the ocean. Active tracking of threshers show they spend daylight hours diving beneath the thermocline, and spend the night time within the mixed layer (Cartamil et al., 2010a, 2016). Spatial proximity to the coast and the quality of their flesh for human consumption exposes *A. vulpinus* to various fisheries. Thresher sharks are captured as bycatch and are targeted for commercial, recreational, and artisanal fisheries (CDFG, 1999; Cartamil et al., 2011).

Thresher shark susceptibility to fisheries became evident in the northeast Pacific Ocean in the late 1980s marked by a reduction in catch rates (Cailliet et al., 1993). At the height of the California drift gill net fishery, approximately 200 boats yielded 2.4 million pounds of thresher shark in 1982; by the late 1980s this yield was less than half, with a similar drop in catch per unit effort (Cailliet et al., 1993). In the northeast Pacific, the Southern California Bight is an important nursery ground for common thresher sharks (Cartamil et al., 2010b). Adults in the region spend most of their time offshore over the continental slope and just beyond (up to approximately 40 km offshore) (Cartamil et al., 2010a), while juveniles are almost entirely distributed over the shelf (offshore shelf averages approximately 10 km from shore) (Cartamil et al., 2010b).

Despite their value and sensitivity to fisheries, genetic understanding of the common thresher shark remains incomplete.

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Table 1
Shark species used in this study with their associated mitogenome size and the references for those data.

Shark species	Order	GenBank ID	Mitogenome size (bp)	Reference
Alopias vulpinus (common thresher shark)	Lamniformes	MF374733	16,712	This study
Alopias superciliosus (bigeye thresher shark)	Lamniformes	KC757415	16,719	Chang et al. (2014b)
Alopias pelagicus (pelagic thresher shark)	Lamniformes	KF020876	16,692	Chen et al. (2013c)
Megachasma pelagios (megamouth shark)	Lamniformes	KC702506	16,694	Chang et al. (2014a)
Mitsukurina owstoni (goblin shark)	Lamniformes	EU528659	17,743	Unpublished
Carcharodon carcharias (white shark)	Lamniformes	KC914387	16,744	Chang et al. (2013a)
Isurus oxyrhinchus (shortfin mako shark)	Lamniformes	KF361861	16,701	Chang et al. (2013b)
Chiloscyllium punctatum (brown-banded bamboo shark)	Orectolobiformes	JQ082337	16,703	Chen et al. (2014c)
Chiloscyllium giseum (grey bamboo shark)	Orectolobiformes	NC_017882	16,755	Chen et al. (2013a)
Chiloscyllium plagiosum (whitespotted bamboo shark)	Orectolobiformes	NC_012570	16,726	Unpublished
Orectolobus japonicas (Japanese wobbegong shark)	Orectolobiformes	KF111729	16,706	Chen et al. (2013b)
Rhincodon typus (whale shark)	Orectolobiformes	KF679782	16,875	Alam et al. (2014)
Pseudotriakis microdon (false catshark)	Carcharhiniformes	AB560493	16,700	Tanaka et al. (2013)
Muselus manazo (starspotted smooth-hound shark)	Carcharhiniformes	AB015962	16,707	Cao et al. (1998)
Scyliorhinus canicula (small-spotted catshark)	Carcharhiniformes	Y16067	16,697	Delarbre et al. (1998)
Scoliodon macrorhynchos (Pacific spadenose shark)	Carcharhiniformes	JQ693102	16,693	Chen et al. (2014b)
Carcharhinus obscurus (dusky shark)	Carcharhiniformes	KC470543	16,706	Blower et al. (2016)
Glyphis glyphis (speartooth shark)	Carcharhiniformes	KF006312	16,702	Chen et al. (2014a)
Galeocerdo cuvier (tiger shark)	Carcharhiniformes	KF111728	16,703	Chen et al. (2013e)
Sphyrna lewini (scalloped hammerhead shark)	Carcharhiniformes	JX827259	16,726	Chen et al. (2013d)

Phylogentic inference of *Alopias* spp. have relied on morphometric features (Shimada, 2005) or only a few (often four) genetic loci, generally from a combination of the nuclear and mitochondrial genomes (Vélez-Zuazo and Agnarsson, 2011). The phylogenetic structure of the *Alopias* genus is inconclusive and dependent on loci used to infer relatedness. Here, we report the complete mitogenome for the common thresher shark. Using all protein coding genes of the mitochondria, we additionally analyzed the placement of *Alopias* spp. to determine whether increasing the amount of genomic data will improve phylogenetic placement certainty among similar shark species.

2. Methods

Genomic DNA was isolated from random shot metagenomic libraries constructed from the skin surface of six individual common thresher sharks (*Alopias vulpinus*). Sample collection was performed by flushing sterile seawater over the skin surface of six common thresher shark individuals, as discussed in Doane et al. (2017), with the purpose of isolating and describing the skin surface microbial communities; but through this process shark cells were obtained and shark DNA was extracted. Genomic material was isolated using a spin-column isolation procedure (Macharey-Nagel, Germany). Whole genome shotgun sequencing was performed using the Personal Genome Machine (PGM) sequencing by Ion Torrent (Thermo Fisher Scientific, MA, USA). *De novo* contig assembly was conducted on pooled (6 metagenomic libraries) shark samples using SPAdes assembler with k-mer word length 55 (Bankevich et al., 2012). The longest contig was subjected to sequence similarity matching using BLASTn (Altschup et al., 1990) against the mitogenome of the pelagic thresher shark (*Alopias pelagicus*; RefSeq NC_022822.1; (Chen et al., 2013c)) with megablast option on NCBI (91% identity, e-value = 0, query coverage 100%). Reads that went into forming this contig (2111 reads) were extracted from the pooled library and re-assembled for improved assembly of the mitogenome using Bowtie (Langmead et al., 2009), a short read aligner.

The mitogenome contig was annotated using Mitos, a web-based program for *de novo* annotation of metazoan mitochondrial genomes (Bernt et al., 2013). Results were confirmed using MitoAnnotator (MitoFish database), a tool specifically for fish mitogenome annotation (Iwasaki et al., 2013). Predicted genes and gene boundaries were compared with *A. pelagicus* mitogenome. In addition, we identified tRNA boundaries using tRNAScan (Lowe and Chan, 2016). We also confirmed the highly variable control region (D-loop) using Sanger sequencing with primers 14700F CCTTATTATCCAAATCCTCACA and 360R TGGCTGGCACGAGATTTAC. Due to the limitations of standard Sanger sequencing, which limits the quality of the sequences after 900 base pairs, primer walking was additionally performed (Siebert et al., 1995) in order to ensure remaining sequenced nucleotides were of high quality.

A comparison of the mitogenome of *A. vulpinus* was made against other shark mitogenomes, including those from the orders Lamniformes (*A. pelagicus*, *A. superciliosus*, *Megachasma pelagios*, *Mitsukurina owstoni*, *Carcharodon carcharias*, and *Isurus oxyrinchus*), Orectolobiformes (*Chiloscyllium punctatum*, *C. griseum*, *C. plagiosum*, *Orectolobus japonicas*, and *Rhincodon typus*) and Carcharhiniformes (*Pseudotriakis microdon*, *Mustelus manazo*, *Scyliorhinus canicula*, *Scoliodon macrorhynchos*, *Carcharhinus obscurus*, *Glyphis glyphis*, *Galeocerdo cuvier*, and *Sphyrna lewini*) to distinguish phylogenetic relatedness. The spinetail devilray (*Modula japanica*) was included as an outgroup. Sequence alignment of all mitogenomes was conducted with MUSCLE (Edgar, 2004) using eight iterations. We determined the best partitioning scheme using PartitionFinder under the Akaike information criterion using a greedy search (Lanfear et al., 2012), with a starting scheme that partitioned by locus and codon position within each locus.

We estimated a maximum likelihood phylogeny using RAxML-HPC v.8.2.9 (Stamatakis, 2014, 2006) in the CIPRES Science Gateway (Miller et al., 2010) using the partitioning scheme output by PartitionFinder, with a separate GTR + Γ model specified for each partition. The default rapid hill-climbing algorithm in RAxML was executed with 10 searches from different random stepwise addition parsimony starting trees. Support values on the best tree were estimated using non-parametric bootstrapping with the auto-MRE automatic stopping criterion. Bayesian phylogeny was estimated using MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012). We used the best partitioning scheme from PartitionFinder with substitution model parameters unlinked across partitions. For each partition, we used the nst = mixed option in MrBayes to marginalize across all subset models of the GTR model using reversible-jump MCMC and accommodated rate heterogeneity among sites under a Γ distribution (Huelsenbeck et al., 2004; Ronquist et al., 2012).

3. Results and discussion

We report the mitogenome for the common thresher shark with 16,712 base pairs, similar to other shark species (true sharks; superorder Euselachii) (Table 1). The *A. pelagicus* and *A. superciliosus* mitogenomes are 16,692 and 16,719 base pairs, respectively. Our contig

Table 2
The number and proportion of each nucleotide in the mitochondrial sequence of *A. vulpinus*.

Base	Count	Percent (%)
A	5401	32.31
T	4989	29.85
G	2188	13.09
C	4134	24.74

containing the mitogenome matched most closely to the pelagic thresher (*Alopias pelagicus*) with 91% exact matching and 99% query coverage. The construction of the *A. vulpinus* mitogenome is the result of 6 pooled shotgun metagenomes. Coverage ranged across the mitogenome with the highest coverage being 48 × and the lowest at 1 ×. A median coverage of 21 × was found at 799 positions.

The control region (D-loop) is 1074 base pairs. Base pair composition of the common thresher shark mitogenome is 32.31% A, 29.85% T, 13.09% G, and 24.74% C. GC of the *A. vulpinus* mitogenome is 37.83% (Table 2). Identified features within the mitogenome include 22 tRNA, two rRNA, 13 protein coding genes, a replication origin (OL) and a control region (D-loop) (Fig. 1; Table 3). The light chain encodes for 7 tRNA genes including; $tRNA^{Gln}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Glu}$, and $tRNA^{Pro}$; and one protein coding gene, NAD6. The heavy chain encodes the remaining 28 of genes, including two rRNA, 15 tRNA, and 12 protein coding regions (Table 3). Protein coding genes all have start codons of ATG, with the exception of the *COI* gene which is GTG, similar to that observed for the pelagic thresher and whale sharks. Stop codons are TAA, except *CYTB* which uses TAG and *NAD6* is AGG, which is similar to *A. pelagicus*. Incomplete stop codons were found for *COII*, *ND3*, and *ND4*, similar to both *A. pelagicus* and *A. superciliosus*. Junctions between genes ranged from no gaps to 13 base pairs. Overlapping gene regions are present, with the greatest overlap being −9 bp between *AP8* and *AP6*. The *A. pelagicus* and *A. superciliosus* also have the greatest overlap between these two proteins, with an overlap of −10 bp (Table 3). The number of genes (rRNA, tRNA, and protein coding) and their order in the *A. vulpinus* mitogenome are similar to other shark species.

We constructed a phylogenetic tree based on 13 protein coding regions from the mitogenome of 20 shark species (superorder Euselachii) and an outgroup species, *Mobula japonica* (superorder Batoidea) (Table 1; Fig. 2). Sharks from three orders were analyzed, including

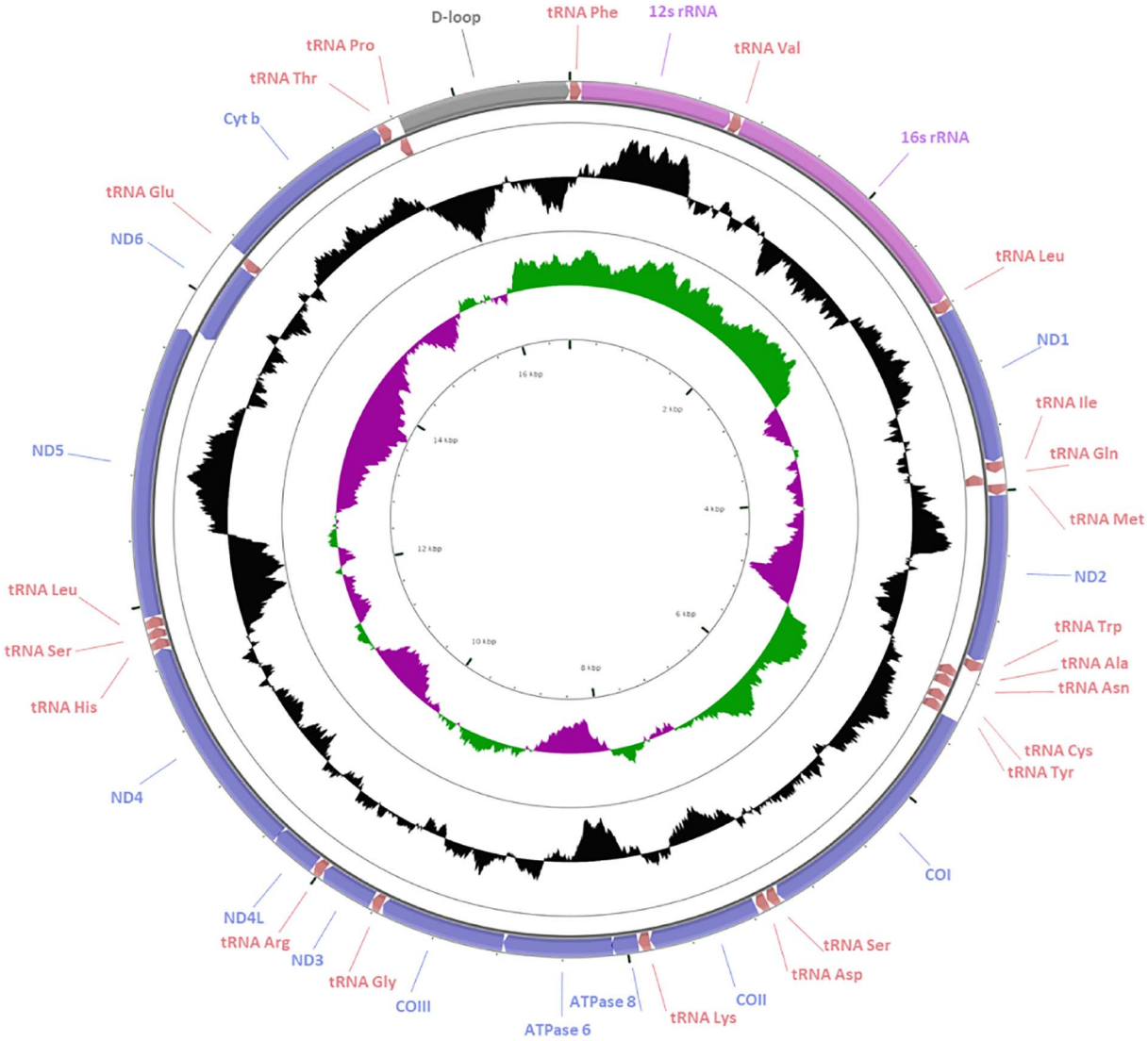

Fig. 1. The alignment of the mitochondria, indicating gene position. Outer circle is aligned genes with outermost representing the heavy strand (H) and adjacent inner ring the light strand (L). Blue represents protein coding regions, red tRNA, purple rRNA, and grey is the D-loop. Middle circle represents GC content: Protruding outwards indicates GC rich while inward protrusion AT rich regions. The inner most circle is GC skew (G − C/G + C) with positive skew (between 0 and 1) represented by green and negative as purple (between 0 and −1).

Table 3
Genes identified in the *A. vulpinus* mitochondrial sequence. H indicates forwards strand and L the reverse strand. Percent similarity is a Blastn comparison of each gene region from *A. vulpinus* to the equivalent in *Alopias pelagicus* (A), *Rhincodon typus* (B), and *Sphyrna lewini* (C).

Name	Start	Stop	Intergenic spacer	Start/stop codon	Strand	Length (bp)	Percent similarity A B C
$tRNA^{Phe}$	1	71	0		H	71	96 87 93
12s rRNA	72	1025	−3		H	954	96 86 87
$tRNA^{Val}$	1023	1094	−0		H	72	93 90 92
16s rRNA	1095	2769	−1		H	1675	95 86 88
$tRNA^{Leu(UAG)}$	2769	2843	0		H	75	97 93 94
ND1	2844	3817	2	ATG/TAA	H	974	90 82 81
$tRNA^{Ile}$	3820	3889	−2		H	70	94 84 90
$tRNT^{Gln}$	3888	3959	0		L	72	93 89 81
$tRNA^{Met}$	3960	4028	0		H	69	96 96 97
ND2	4029	5071	0	ATG/TAA	H	1043	91 78 78
$tRNC^{Trp}$	5072	5142	1		H	71	94 92 99
$tRNG^{Ala}$	5144	5212	0		L	69	97 97 91
$tRNT^{Asn}$	5213	5285	0		L	73	99 95 89
O_L	5286	5318	0		–	33	
$tRNC^{Cys}$	5319	5385	1		L	67	94 90 85
$tRNT^{Tyr}$	5387	5457	7		L	71	94 89 90
COI	5465	7011	3	GTG/TAA	H	1547	91 84 85
$tRNA^{Ser(GCU)}$	7015	7085	4		L	71	97 84 88
$tRNA^{Asp}$	7090	7159	7		H	70	97 80 87
COII	7167	7858	−1	ATG/T-	H	692	94 87 87
$tRNA^{Lys}$	7858	7931	1		H	74	99 93 91
ATP8	7933	8099	−9	ATG/TAA	H	167	90 80 76
ATP6	8091	8773	0	ATG/TAA	H	683	90 81 82
COIII	8774	9558	3	ATG/TAA	H	785	92 85 87
$tRNA^{Gly}$	9562	9631	0		H	70	96 94 90
ND3	9632	9980	0	ATG/T-	H	249	90 77 83
$tRNA^{Arg}$	9981	10,050	0		H	70	97 96 91
ND4L	10,051	10,346	−6	ATG/TAA	H	296	87 75 80
ND4	10,341	11,720	1	ATG/T-	H	1380	90 78 81
$tRNA^{His}$	11,722	11,790	0		H	69	97 94 94
$tRNA^{Ser(UGA)}$	11,791	11,856	0		H	66	99 99 92
$tRNA^{Leu(UAA)}$	11,857	11,928	9		H	72	100 96 96
ND5	11,938	13,743	13	ATG/TAA	H	1806	88 80 81
ND6	13,757	14,274	1	ATG/AGG	L	518	89 77 80
$tRNA^{Glu}$	14,276	14,345	−1		L	70	91 93 94
Cyt b	14,354	15,492	0	ATG/TAG	H	1139	89 81 81
$tRNA^{Thr}$	15,493	15,566	2		H	74	92 86 87
$tRNA^{Pro}$	15,569	15,637	0		L	69	100 96 94
D-loop	15,638	16,712			–	1075	89 84 68

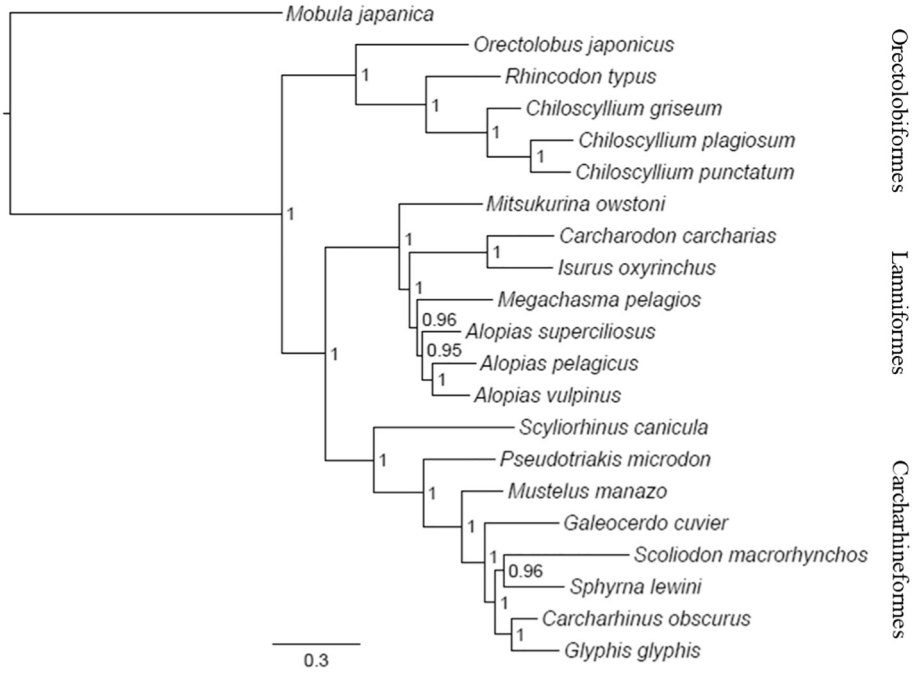

Fig. 2. Bayesian phylogenetic estimate for the relationships among shark species from three Orders and an outgroup species from Rajiformes using the 13 protein-coding loci from the mitogenome. Numbers at nodes are posterior probabilities.

Lamniformes, Orectolobiformes, and Charcharhiniformes. The relationship of these three orders has been inconclusive (Vélez-Zuazo and Agnarsson, 2011). Depending on molecular markers used, the Order Lamniformes is either monophyletic to Carcharhiniformes with Orectolobiformes as an outgroup (Heinicke and Naylor, 2009) or monophyletic with Orectolobiformes and Carcharhiniformes as an outgroup (Vélez-Zuazo and Agnarsson, 2011). Our analysis, based on 13 protein coding mitochondrial genes, indicates that the three orders form distinct groupings, with Lamniformes and Carcharhiniformes forming a clade sister to Orectolobiformes (posterior probability 1; Fig. 2). These results contrast to Alam et al. (2014) who similarly analyzed the protein coding genes of the mitochondrion, but using fewer species. Alam et al. (2014) reported that Lamniformes and Orectolobiformes are monophyletic. The analysis presented here, using the same 13 protein coding mitochondrial genes, indicate Lamniformes and Carcharhiniformes are monophyletic. Our results are congruent with other molecular studies analyzing a reduced set of genes originating from both mitochondrial and nuclear genomes (4: *CYTB*, *NADH2*, *NADH4*, and *RAG1*) (Naylor et al., 2005; Heinicke and Naylor, 2009) (3: *12S*, *16S*, and *RAG1*) (Naylor et al., 2005). Within Lamniformes, the three *Alopias* spp. group together. Our results agree with Vélez-Zuazo and Agnarsson (2011), that *A. vulpinus* and *A. pelagicus* underwent the most recent divergence, with *A. superciliosus* forming an outgroup. In addition, we found that the inclusion of *A. vulpinus* protein coding mitochondrial genes increased support for the placement of *A. vulpinus* and *A. pelagicus* together, indicated by *posterior* probability of 1 (Fig. 2).

4. Conclusion

The mitogenome of the common thresher shark is similar in length to other *Alopias* spp. having 16,712 base pairs. The mitochondrial features present and their arrangement are typical of vertebrate mitogenomes being circular with 13 protein coding regions, two rRNA, 22 tRNA, a replication region and a control region. Using the whole mitogenome, we provide greater certainty for relationships among *Alopias* spp. By comparing our results with those of other studies that analyze a reduced number of gene regions, we can inform the best combination of genes for assessing phylogenetic relationships. The construction and analysis of whole genomes is computationally expensive, therefore validating those gene regions appropriate for phylogenetic assessment is imperative for future studies. Thresher sharks are an important fisheries species, thus increasing population resolution is critical for effective management. Complete sequencing of the mitogenome of *A. vulpinus* has provided the initial steps towards resolving the population boundaries of *A. vulpinus* along the northeastern Pacific coast. In addition, we present an alternative means of gaining access to the host's genetic information from datasets collected for microbiome research. With increasing numbers and types of DNA datasets becoming publicly available, data mining for host's genes provides an additional approach to studying aspects of host population dynamics and phylogenetic inference.

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