Mitogenomic characterization and phylogenetic position of the oldest living vertebrate species - the Greenland shark (*Somniosus microcephalus*)

—

Aintzane Santaquiteria Gil
BIO-3950 Master thesis in Biology, July 2016

© Julius Nielsen

Acknowledgments

First of all, I would like to thank my two supervisors; Kim Præbel and Julius Nielsen. Thanks for giving me the opportunity to work with this big and fabulous animal, a dream that you made happen. Awesome expeditions, thanks to The Arctic University of Norway (UiT), Andørja Adventures company and the "Old and cold – Biology of the Greenland shark" project from the University of Copenhagen, UiT, University of Indiana South Ben (U.S.A.) and Greenland Institute of Natural Resources. I will never forget the first time I saw a Greenland shark, almost 4 meters long, incredible. Thank you for steering me on the right direction to become a scientist. The door of your offices were always open whenever I ran into a trouble or had a question. But especially, thanks for the useful advices and comments through the learning process of my master thesis.

Besides my supervisors, I would like to thank Tanja, the lab technician with endless lab skills, if you have any doubt, go and ask her and everything will be under control. Thanks for helping me with all the long days in the lab. Thanks also goes to Shripathi, his brilliant bioinformatics skills helped me to achieve the best of my results. I would also like to express my gratitude to the people working at the sequencing facility of the Medicine faculty and the Barents BioCenter at UiT for sequencing of the samples.

Thank you so much to my friends in Tromsø, that motivated me making long days easier. The same goes to my friends from Pamplona and surroundings, always ready to help me forget my worries. A special thanks to Renee and Patricia for supporting me and being there all the time.

And last but not least, thank you to my lovely parents and sister, for providing me support and continuous encouragement through my years of study. This accomplishment would not have been possible without their effort.

Eskerrik asko!

Tromsø, July 2016
Aintzane Santaquiteria Gil

Preface

This thesis concludes my Master of Science education in Biology, with a specialization in population genetics at the Arctic University of Norway (UiT) in Tromsø.

The research is part of the Greenland shark project, a collaboration between UiT, University of Copenhagen, University of Indiana South Ben (U.S.A.) and Greenland Institute of Natural Resources. The project has been supervised by the associated professor Dr. Kim Præbel from UiT and PhD student Julius Nielsen from the University of Copenhagen. The aim of the project was to characterize the mitogenome of the Greenland shark and to accurately place the Greenland shark in the phylogenetic tree of the sharks. To solve this aim, several fieldworks were carried out in the eastern Greenland and northern Norway, catching sharks and collecting tissue samples from the dorsal fin.

Being part of this project has helped me develop my career as a scientist. Field and laboratory experiences had increased my motivations to continue doing research. Especially when genetics, evolution and conservation of a species are combined, as it was done in this project.

The thesis was written as a longer draft for a scientific article for the purpose of submitting it for peer-review in a scientific journal.

Table of contents

Abstract

The Greenland shark (Squaliformes, *Somniosus microcephalus*) is the largest fish living in Arctic waters, but little is known about its biology. This species lives for at least 272 years and is listed as a near threatened species on the IUCN´s Red list of Threatened Species. As *S. microcephalus* is the oldest living vertebrate species, it is important to strive for its conservation. The aim of the study was to sequence and provide the first characterization of the *S. microcephalus* mitogenome, in order to accurately determine the phylogenetic position of this elusive species. Mitochondrial DNA (mtDNA) is a widely used tool for phylogenetic analysis, as it is not subjected to recombination (maternal inheritance) and is relatively easy to amplify. Using next generation sequencing, the size of the *S. microcephalus* mitogenome was estimated to 16,730 bp. The mitogenome was composed by 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes, and a control region (D-loop). This composition resembles what have been observed for other vertebrate mitogenomes. In the comparative phylogenetic analysis based on the mitogenomes of 17 related shark species, S. *microcephalus* was positioned as a sister species of the Pacific sleeper shark (*Somniosus pacificus*). The single genes provided more incongruent topologies for phylogenetic reconstructions than when the mitogenome was used. Divergence time estimates confirmed that *S. microcephalus* and *S. pacificus* diverged 3.5 million years ago (Mya). Less than 1 % of nucleotide difference and a recent indication of gene flow between these close related species, suggested to be a single species. The results suggested a possible continuous distribution of the *Somniosus* subgenus (*S. microcephalus, S. pacificus* and *S. antarcticus*) across the globe. The availability of *S. microcephalus* mitogenome will contribute to aid further studies of phylogeography, population structure and conservation genetics in this species and sleeper sharks in general.

Introduction

The Greenland shark, *Somniosus microcephalus* (Bloch & Schneider, 1801), belongs to the family Somiosidae, within the order Squaliformes of the class Chondrichthyes. There are two other species within this subgenus, *Somniosus*, Pacific sleeper shark (*Somniosus pacificus*) and Antarctic sleeper shark (*Somniosus antarcticus*). The *S. microcephalus* have a large distribution area which encompass the Arctic Ocean as well as the North Atlantic Ocean at all depths, and the deep ocean water masses in the entire Atlantic Ocean. Individuals have been observed in waters offshore Norway, Svalbard, Iceland, Greenland, Baffin Island, Eastern Quebec, Newfoundland and Nova Scotia (MacNeil et al., 2012). Punctual individuals were recorded at the Azores islands (Quero et al., 1982) and the Gulf of Maine, USA (Bigelow & Schroeder, 1948). Also some observations have been made in the Gulf of Mexico at a depth of 1,749 m (Deep-C, 2013). However, still little is known about the largest fish living in Arctic waters.

There are no abundance estimates of *S. microcephalus*, although they are commonly observed in the North Atlantic Ocean by fishermen, sealers and researchers (Dunbar & Hildebrand, 1952; Templeman, 1963). According to IUCN´s list (Red List of Threatened Species), *S. microcephalus* is listed as near threatened (NT) species due to the possible population declines from fishing pressure and limiting life history characteristics (Kyne et al., 2006). A lifespan of at least 272 years, make them the longest living vertebrate known in science (Nielsen et al., in press). Furthermore, the females, on average larger and heavier than males, reach sexual maturation at an age of at least ~130 years (Nielsen et al., in press) and > 400cm total length (Lt) (Yano et al., 2007). The diet of this shark is known to be widely diverse, including different species of gastropods, cephalopods, crustaceans, fish and mammals (Yano et al., 2007; McMeans et al., 2010; Leclerc et al., 2012; Nielsen et al., 2014). Little is known about how they catch their prey and whether they are scavengers or active predators, but it has been suggested that they are both (Leclerc et al., 2012; Nielsen et al., 2014). Thus, *S. microcephalus* as an animal with a high trophic position (Hobson et al., 2002) can be an important key to the trophic dynamics in the Arctic marine ecosystems.

Fragments of the mtDNA are a commonly used tool for phylogenetic analysis (Moritz, 1994), since the mtDNA is relatively easy to amplify and maternally inherited (non-recombining) (Gilbert et al., 2008; Singh et al., 2009). Nowadays, the use of the mitogenome

is becoming more frequent (Strohm et al., 2015). The maternal inheritance pattern, makes the mitogenome a suitable genetic marker for inferring phylogenies, due to the linkage of the mtDNA mutations can estimate the evolutionary histories, showing the origins of maternal lineages between and within species (Avise et al., 1987). The mitogenome can estimates divergences occurred more than few million years ago (Curole & Kocher, 1999). Owing to the high cost for sequencing a complete mitogenome, scientists have used single genes (e.g. control region or cytochrome *b*) or small portion sequences (Galtier et al., 2009; Jacobsen et al., 2012) of the mitogenome for phylogenetic analysis. Although, when species have a recent or rapid divergence, single genes may not provide enough information for phylogenetic analysis as the complete mitogenome does (Jacobsen et al., 2012). This may be especially the case in chondrichthyes, where the mitochondrial genes have low evolutionary rates (Martin et al., 1992; Renz et al., 2013).

Previous studies, using a ~703 bp fragment of the cytochrome *b* (Cyt *b*) have shown that *S. microcephalus* is placed as a sister species of *S. pacificus* and *S. antarcticus* in the Squaliformes order, within the phylogenetic tree of sharks based on a Bayesian Inference (Vélez-Zuazo & Agnarsson, 2011) and a maximum likelihood (ML) analysis (Sorenson et al., 2014). Sorenson et al. (2014) conducted a time-tree by estimating divergence times of modern sharks, based on fossil calibrations using BEAST v.1.6. computer program (Drummond & Rambaut, 2007). They concluded that Squaliformes originated in the Late Triassic (216 Mya), although their crown age was dated to the Middle Jurassic (162 Mya). Regarding to *Somniosidae* family, the divergence between *S. microcephalus* and, *S. pacificus* and *S. antarcticus* occurred more recently, less than 10 Mya (Sorenson et al., 2014).

Murray et al. (2008) analyzed the variations of 703 bp Cyt *b* sequences in three sleeper sharks; *S. microcephalus*, *S. pacificus* and *S. antarcticus*. They conducted a minimum spanning parsimony network between the three species from four different locations; *S. microcephalus* from the North Atlantic (Iceland and Cumberland Sound), *S. pacificus* from the Pacific Ocean (Taiwan and Alaska) and *S. antarcticus* from the Southern Ocean (Antarctic). Two different clades were found where *S. microcephalus* formed one clade and *S. pacificus* and *S. antarcticus* formed the other. The network showed that from the 21 haplotypes identified, the *S. pacificus* and *S. antarcticus* were sharing four haplotypes, while *S. microcephalus* were not sharing any. Moreover, the analysis of molecular variance (AMOVA) showed evidence for genetic structuring between the two clades but not between *S. pacificus* and *S. antarcticus*. Based on

nucleotide substitutions, Murray et al. (2008) estimated that *S. microcephalus* diverged from *S. pacificus* and *S. antarcticus* 3.5 Mya. Therefore, they suggested that *S. microcephalus* is a separate species from *S. pacificus* and *S. antarcticus*, and that the separation of *S. pacificus* and *S. antarcticus* was not supported by the Cyt *b* analysis (Murray et al., 2008). A recent study on *S. microcephalus* juveniles in the Canadian Arctic have shown, based on genetic diversity, a total of 11 haplotypes, of which four of them were previously described by Murray et al. (2008). Genetic analysis, using a 702 bp sequence of the Cyt *b*, detected for the first time a *S. pacificus* haplotype (H4) in a *S. microcephalus* individual caught outside the Pacific Ocean. The study identified two *S. microcephalus* individuals carrying the H4 haplotype, which made Hussey et al. (2014) to suggest that *S. microcephalus* and *S. pacificus* hybridize.

Sequencing the complete mitochondrial genome would give more consistent inference of the phylogenetic position (Alam et al., 2014), and provide a needed tool for further genetic studies of the elusive species. Therefore, the objectives of this study were to: 1) sequence, validate, and characterize the mitogenome of *S. microcephalus* to provide a reference mitogenome, 2) use the available mitogenomes from other shark species to obtain the phylogenetic position of *S. microcephalus* and discuss the strength and weaknesses of single genes vs mitogenomes for inferring phylogenetic positions, 3) estimate, using the mitogenomic variation, when *S. microcephalus* diverged from *S. pacificus* and provide a calibrated estimate of divergence within the chondrichthyes and 4) evaluate the species status of the *S. microcephalus* and *S. pacificus*. By solving these objectives, the study will provide a solid basis for understanding more about the biology of this elusive species. The mitogenomic resource will enable future phylogeographical and population genetic studies. Therefore, in this study, it was hypothesized that 1) the *S. microcephalus* mitogenome should have similar structure as documented in other elasmobranchs. 2) the divergence between *S. microcephalus* and *S. pacificus* should correlate with the previously reported geological event, the Isthmus of Panama. 3) The mitogenomic data of *S. microcephalus* and *S. pacificus* should show that they are two different species.

Material and methods

One female and one male *S. microcephalus* (*GS53* and *GS88* respectively) were caught by long-line in Greenland waters (Table 1). The female, 445 cm long, was caught offshore the South-east of Greenland in 2013, while the male, 306 cm long, was caught in 2014, in Disko Bay, located North-west of Greenland. Tissue samples were obtained from white muscle tissue and preserved in tubes with 96 % ethanol at -20 ˚C until extraction of DNA.

Table 1 Individuals used to infer the complete mitochondrial DNA genome.

ID	TL (cm) [a]	Sex	Area	Region	Year
GS53	445	F	Offshore	SE, Greenland	2013
GS88	306	M	Disko Bay	NW, Greenland	2014

[a] *TL = total length.*

Laboratory procedures

Mitogenome sequencing

DNA was extracted using the Salt-lysis method described by Aljanabi & Martinez (1997). The method involves disruption and lysis of the tissue, followed by the removal of proteins, RNA and other contaminants with a final recovery of the DNA. Small modifications were done in the protocol, where the DNA dry pellets were dissolved in a 50 µl Elution buffer (E.Z.N.A.® Tissue DNA Kit, OMEGA Bio-tek). The quality, purity and concentration of the extracted DNA was determined with a nanodrop spectrophotometer (ND-1000 v.3.7 User´s manual) and agarose gel electrophoresis (0.8 % agarose, 80 V and 25 min) (Barril & Nates, 2012).

To design species-specific primers for the long-range polymerase chain reaction (long-range PCR), mitogenome sequences from the GenBank database of the two closest species, *S. pacificus* (GenBank Accession number: NC_022734.1) and *Squalus acanthias* (GenBank Accession number: NC_002012.1), were aligned using MEGA6 software (Tamura et. al., 2013). To identify potential primer sets, conservative regions were identified in the alignment. Each primer was checked in Primer3 (Rozen & Skaletsky, 2000) to observe whether they fulfill the optimal characteristics required for a success long-range PCR; such characteristics were:

length (27 - 34 bp), Tm (65 - 75 ºC) and GC content (40 - 60 %). A total of seven primer sets were designed to amplify the complete mitogenome in three overlapping fragments. Among these primer sets, three were chosen as they provided consistent amplification of products with the correct size (Table 2 and Fig. 1).

The long-range PCR (See protocol in Appendix I) was performed using a reaction mixture consisting of 15 µl of Phusion Hot Start II High-Fidelity PCR Master Mix polymerase (Thermo Scientific), 3 µl of each primer (5 µM), 6 µl of H_2O and 3 µl of DNA template, in a 30 µl volume. The PCR program consisted of an initial denaturing at 98 ˚C for 30 s; 35 cycles at 98 ˚C denaturing for 7 s, 71 ˚C annealing for 20 s, and 72 ˚C extension for 2 min; followed by a final extension step at 72 ˚C for 7 min. The success of the PCR amplifications was confirmed by 0.8 % agarose gel electrophoresis. To confirm the size of obtained PCR products, a 2 kb ladder (BioLabs) was included as a standard in each electrophoresis. The PicoGreen dsDNA Quantification assay (PicoGreen® dsDNA Quantification Reagent and Kits, Molecular Probes) was used to estimate the DNA concentration (ng/µl) of each amplicon.

The three amplicons from each individual were subsequently pooled in equimolar concentrations and sent for purification and next generation sequencing (MiSeq, Illumina) at the Barents BioCenter (BBC), UiT. The amplicons pools were individually barcoded and sequenced using paired-end sequencing (300 bp read-length) in a single lane. The mentioned procedures are showed as a workflow in the Appendix II.

Table 2 Primer sets designed for amplifying the complete mitogenome in three amplicons.

Primer set	Primer name	Sequence	Base length	Tm °C	GC %	Amplicon length
1	Som_F1	5´-GATACCCTACTATGCCCAACCACAAACTTAGAC-3´	33	68.4	45.5	7431
	Som_R1A	5´-GCATATCACTAAGGGTGGTAGGGAGTCA-3´	28	67.1	50.0	
2	Som_F2A	5´-AGATGCAGCCTCCCCAGTTATGGAAGAAC-3´	29	72.0	51.7	5499
	Som_R2	5´-TAGAGTGGAGTAGGGCAGAGACTGGCGT-3´	28	70.8	57.1	
3	Som_F3	5´-CTCTTGGTGCAACTCCAAGCAAGAGCTATGA-3´	31	72.9	48.4	6128
	Som_R3	5´-GATGCAAAAGGTACGAGGTTGAGTCTCTGC-3´	30	71.0	50.0	

Mitogenome assembly and validation

The de-multiplexing and filtering of the Illumina short reads and the initial assembly of the mitogenomes were performed at BBC. The two received draft mitogenomes were then aligned against the closest relative species, *S. pacificus*, using MEGA6 software (Tamura et al., 2013), to detect SNPs, gaps, inserts and deletions. To verify the draft mitogenomes, seven new primer sets were designed for Sanger sequencing in order to re-sequence the most discordant regions. To design the seven primer sets, the two draft mitogenomes were alignment in MEGA6 software (Tamura et al., 2013) and primer sets were checked in Primer3 (Rozen & Skaletsky, 2000) (Table 3 and Fig. 1).

Table 3 Primer sets designed for amplifying the discordant regions of the assembled mitogenomes.

Primer Set	Primer name	Sequence	Base length	Tm °C	GC %	Amplicon length
a	P1_F	5´-AGACTTCCGAGTAGCGGTGA-´3	20	60.0	55.0	451
	P1_R	5´-ATGTCCTCAGTTTGGGTTCG-´3	20	60.0	50.0	
b	P2_F	5´-CCCACTTGCCTACATTGTCC-´3	20	60.4	55.0	536
	P2_R	5´-TTATGGCTAATGGTCAGCCTG-´3	21	60.1	47.6	
c	P3_F	5´-CCGTTTGATCTAACCGAAGG-´3	20	59.6	50.0	752
	P3_R	5´-TGGTTGCTTCTACTGCTCGG-´3	20	61.5	55.0	
d	P4_F	5´-TGCCGGAGTTAACCTAACCTTC-´3	22	61.7	50.0	699
	P4_R	5´-CGTAAAGATGGGAGGGCAAT-´3	20	61.2	50.0	
e	P5_F	5´-CCGATTGCCCTATACGTAACC-´3	21	60.6	52.4	766
	P5_R	5´-TGAGCCTGAACAGAGGAAAAG-´3	21	59.6	47.6	
f	P6_F	5´-TCCTTCACCGCCATTTACAG-´3	20	61.0	50.0	516
	P6_R	5´-ATCAGTCCTTGTTGGGGTTG-´3	20	59.8	50.0	
G	P7_F	5´-TGGCTCCAACAACCCAATAG-´3	20	60.9	50.0	782
	P7_R	5´-ATCAGGTGAAGTTGTATGTGGC-´3	22	59.0	45.5	

Standard PCR (F+R) was performed for both individuals, *GS53* and *GS88* using a reaction mixture consisting of 0.6 µl of DyNAzyme^{MT} EXT DNA Polymerase (Thermo Scientific), 1 µl for each primer (5 µM), 2 µl of 10X Optimized DyNAzyme^{TM} EXT Buffer (Thermo Scientific), 0.6 µl of dNTP (10 µM), 10.8 µl of H_2O and 4 µl of DNA template, in a 20 µl volume. The PCR program consisted of an initial denaturing at 94 ˚C for 5 min; 35 cycles at 94 ˚C denaturing for 30 s, 59 ˚C annealing for 35 s, and 72 ˚C extension for 40 s; followed by a final extension step at 72 ˚C for 10 min. The success of the PCR amplifications was confirmed by 1 % agarose gel electrophoresis. To confirm the size of the obtained PCR product, a 100 bp ladder (BioLabs) was included as a standard in each electrophoresis.

To prepare the amplified products for the BigDye reaction, unincorporated primers and nucleotides from the PCR reactions were removed by enzyme digestion using Illustra^{TM} ExoStar^{TM} 1-Step protocol (GE Healthcare Life Sciences). For each sample, 1.5 µl of illustra ExoStar 1-step product was mixed with 4 µl PCR product obtained by the standard PCR, and incubated for 15 min at 37 ˚C and 15 min at 80 ˚C.

BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used after purifying the PCR products to prepare the samples for Sanger sequencing. The BigDye reaction mixture consisted of 0.5 µl of BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems), 0.5 µl of primer (3.5 µM), 1µl of BigDye Terminator v3.1 5X Sequencing Buffer (Applied Biosystems), 2 µl of H_2O and 1µl purified of DNA template, in a 5 µl volume. The sequencing reactions consisted of an initial denaturing at 96 ˚C for 1 min; 35 cycles at 96 ˚C denaturing for 10 s, 50 ˚C annealing for 5 s, and 60 ˚C extension for 4 min. Finally, all the samples were delivered for purification and Sanger sequencing at the sequencing facility at the Medicine faculty at UiT. The mentioned procedures are showed as a workflow in the Appendix III.

The obtained sequences from the Sanger sequencing were aligned against the two previously assembled mitogenomes using MEGA6 software (Tamura et. al., 2013) and the complete reference mitogenome of *S. microcephalus* was validated by manual verification of SNPs, gaps, inserts and deletions. Finally, the validated genome was annotated using MitoAnnotator, a tool from MitoFish web-database (Iwasaki et al., 2013).

Data analysis

Phylogenetic analysis

Phylogenetic analysis was performed to position *S. microcephalus* among the other elasmobranchs. The assembled and annotated mitogenome of *S. microcephalus* was compared against 17 mitogenomes available in GenBank of related shark species from the orders Squaliformes, Squatiniformes, Pristiophoriformes, Heterodontiformes, Lamniformes and Hexanchiformes (Table 4). Two species of Batoidea (skates and rays) from the orders Myliobatiformes and Rajiformes and three Holocephali (chimaeras) from Chimaeriformes were selected as outgroups (Table 4). This comparison was made using the CGView comparison tool (CCT), that used BLAST to conduct the comparison of the *S. microcephalus* reference genome with the rest of species mitogenomes showing the results as a circular map (Grant et al., 2012). To perform the subsequent phylogenetic analysis, all the sequences were aligned using the *Muscle* application with standard setting of MEGA6 software (Tamura et al., 2013). But first, in order to have more detailed comparisons, nucleotide similarities between all species were compared using Geneious v.9 (http://www.geneious.com, Kearse et al., 2012).

To obtain the best evolutionary model of nucleotide substitution for the aligned species, the Likelihood Scores and the Akaike information criterion (AIC) were calculated and evaluated using jModeltest v.2.1.7 (Guindon & Gascuel, 2003; Darriba et al., 2012). GTR + I + G resulted as the most probable model for AIC (Akaike information criterion). To infer a Bayesian phylogenetic tree of aligned species, Bayesian Inference analysis was conducted in MrBayes v.3.2 (Ronquist et al., 2012). Markov chain Monte Carlo (MCMC) analysis was run for 50,000 generations for each 500 samples with a 25 % burn-in, until converge of < 0.01. Two chains were used with a heating parameter of 0.1. FigTree v.1.4.3 software (Rambaut, 2005-2016) was used to annotate the Bayesian tree. To confirm the topology and phylogenetic relationships between the different species obtained in Bayesian tree, neighbor joining (NJ) and maximum likelihood (ML) trees were made in MEGA6 software (Tamura et al., 2013) with 1000 bootstrap replicates. NJ analysis was made using Maximum Composite Likelihood model, including nucleotide transitions and transversions substitutions. ML analysis was computed using the substitution model GTR + I + G, and Nearest Neighbor Interchange (NNI) approach as a maximum likelihood heuristic method to search for tree topology.

To evaluate the strength and weakness of using single mitochondrial genes or mitogenomes for inferring phylogenetic positions, the control region (or D-loop) and the COI genes were analyzed from all selected species. Best fitting model was TVM + I + G for control region and TIM2 + I + G for COI using AIC score (Guindon & Gascuel, 2003; Darriba et al., 2012). Bayesian Inference analyses were computed by MrBayes v.3.2 (Ronquist et al., 2012) for the COI region using the same settings as the complete mitogenome; while 1,000,000 generations for each 1000 samples were used for the control region.

To estimate the divergence times within chondrichtyes, a calibrated time-tree was inferred using the already obtained ML tree as the start tree. Chimaeras (*Callorhinchus callorynchus*, *Callorhinchus. milii*, *Chimaera monstrosa*) were selected as outgroups for these analyses. Several fossil records were used to calibrate the time-tree (Appendix IV). In order to estimate when the *S. microcephalus* diverged from *S. pacificus*, two different geological events approaches were used for calibration; the closing of the Isthmus of Panama 3.1 - 3.5 Mya (Coates & Obando, 1996; Coates et al., 2004) and the first opening of the Bering strait 4.8 - 5.5 Mya (Gladenkov et al., 2002).

Table 4 Chondrichthyes species (sharks, rays and chimaeras) for comparing *S. microcephalus* mitogenome. For each species, the GeneBank ID and the size of the mitogenome is provided. The species are organized by orders.

Chondrichthyes species	Order	GeneBank ID	Mitogenome size (bp)	Reference
Somniosus microcephalus (Greenland shark)	Squaliformes	-------	16,730	This study
Somniosus pacificus (Pacific sleeper shark)	Squaliformes	NC_022734.1	16,730	Tanaka et al. (2013)
Squalus acanthias (Spiny dogfish)	Squaliformes	NC_002012.1	16,738	Rasmussen & Arnason (1999)
Squalus montalbani (Philippines spurdog)	Squaliformes	NC_028537.1	16,555	Kemper & Naylor (2015)
Cirrhigaleus australis (Southern Mandarin dogfish)	Squaliformes	NC_024059.2	16,544	Yang et al. (2014)
Squatina formosa (Taiwan angelshark)	Squatiniformes	NC_025328.1	16,690	Corrigan et al. (2014)
Squatina japonica (Japanese angelshark)	Squatiniformes	NC_024276.1	16,689	Chai et al. (2014)
Squatina nebulosa (Clouded angelshark)	Squatiniformes	NC_025578.1	16,698	Gao et al. (2014)
Pristiophorus japonicus (Japanese sawshark)	Pristiophoriformes	NC_024110.1	18,430	Tanaka et al. (Unpublished)
Heterodontus francisci (Horn shark)	Heterodontiformes	NC_003137.1	16,708	Arnason et al. (2001)
Heterodontus zebra (Zebra bullhead shark)	Heterodontiformes	NC_021615.1	16,720	Chen et al. (2014)
Hexanchus griseus (Bluntnose sixgill shark)	Hexanchiformes	NC_022732.1	17,223	Tanaka et al. (2013)
Notorynchus cepedianus (Broadnose sevengill shark)	Hexanchiformes	NC_022731.1	16,990	Tanaka et al. (2013)
Hexanchus nakamurai (Bigeyed sixgill shark)	Hexanchiformes	NC_022733.1	18,605	Tanaka et al. (2013)
Alopias pelagicus (Pelagic thresher shark)	Lamniformes	NC_022822.1	16,692	Chen et al. (2013)
Alopias superciliosus (Bigeye thresher shark)	Lamniformes	NC_021443.1	16,719	Chang et al. (2013)
Carcharias taurus (Sand tiger shark)	Lamniformes	NC_023520.1	16,773	Chang et al. (2014a)
Pseudocarcharias kamoharai (Crocodile shark)	Lamniformes	KM597489.1	16,688	Chang et al. (2014b)
*Mobula japanica (*Spinetail Devil ray)	Myliobatiformes	NC_018784.1	18,880	Poortvliet & Hoarau (2013)
Amblyraja radiata (Thorny skate)	Rajiformes	NC_000893.1	16,783	Rasmussen & Arnason (1999)
Callorhinchus callorynchus (Elephantfish)	Chimaeriformes	NC_014281.1	16,758	Inoue et al. (2010)
Callorhinchus milii (Australian ghostshark)	Chimaeriformes	NC_014285.1	16,769	Inoue et al. (2010)
Chimaera monstrosa (Rabbit fish)	Chimaeriformes	NC_003136.1	18,580	Arnason et al. (2001)

Results

Characterization of the *S. microcephalus* mitogenome

The complete sequence of *S. microcephalus* mitochondrial genome was determined by next-generation sequencing (NGS) and verified by Sanger sequencing. The assembled mitogenome was 16,730 bp and composed by 13 protein-coding genes, 2 rRNA (12S rRNA and 16S rRNA) genes, 22 tRNA genes and a control region (Table 5 and Fig. 1). The mitogenome has a GC content of 39.4 % and consist of 30.8 % (A), 29.9 % (T), 14.5 % (G) and 24.9 % (C).

The majority of the protein-coding genes, were transcribed from the heavy (H) strand (ND1, ND2, COI, COII, ATP8, ATP6, COIII, ND3, ND4L, ND4, ND5, Cyt *b*), with the exception of ND6 that was transcribed form the light (L) strand. The usual start codon ATG appeared in all the coding genes except in COI gene, having GTG as the start codon. Seven of the 13 protein-coding genes have TAA as stop codon (COI, ATP8, COIII, ND3, ND4L, ND5 and Cyt *b*), while three genes have incomplete stop codons, either TA (ND2 and ATP6) or T (COII and ND4). Finally, for ND1 and ND6 genes the complete stop codon was TAG. Eight of 22 tRNAs (tRNA^{Gln}, tRNA^{Ala}, tRNA^{Asn}, tRNA^{Cys}, tRNA^{Tyr}, tRNA$^{Ser(UGA)}$, tRNA^{Glu} and tRNA^{Pro}) were encoded by the L strand, whilst the remaining tRNAs were transcribed by the H strand. The control region is located between the tRNA^{Pro} and tRNA^{Phe} genes, with a size of 1075 bp and a GC content of 35.25 % (Table 5, Fig. 1). Not all the mitogenome is covered by genes, several nucleotide gaps were found between adjacent genes (e.g. space of 2 nucleotides between ND1 and tRNA^{Ile}; 4 between tRNA^{Asp} and COII). Beside these gaps, also called intergenic spacers, three pairs of genes were sharing nucleotides; ATP8 and ATP6 had an overlap of 10 nucleotides, ND4L and ND4 an overlap of 7 nucleotides and ND5 and ND6 an overlap of 4 nucleotides (Table 5).
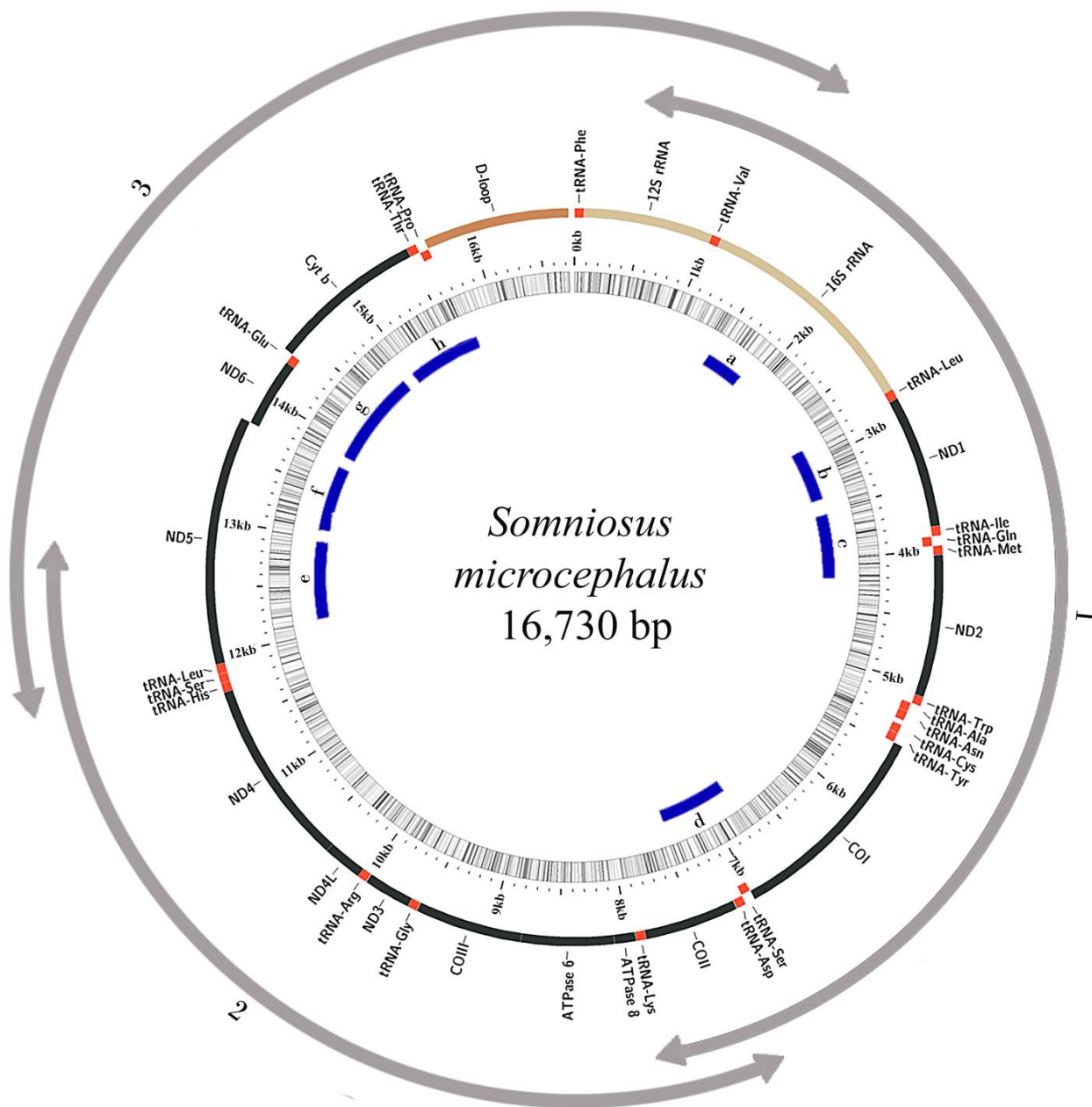
Figure 1 Mitogenome map made by MitoAnnotator showing the gene arrangement of the *Somniosus microcephalus* reference mitogenome. Primer sets used for amplify fragments of the mitogenome by NGS (Table 2) and Sanger sequencing (Table 3) are represented with numbers and grey arrows, and letters and dark blue bars, respectively. The inner circle shows GC content. Number of base pairs (in Kb) are proportional to the length of the mitogenome.

Table 5 Gene arrangement and location in the *Somniosus microcephalus* mitogenome.

Gene	Strand[a]	Gene					Intergenic spacer[c]
		From (bp)	To (bp)	Size (bp)	Start codon	Stop codon [b]	
tRNA[Phe]	H	1	69	70			
12S rRNA	H	70	1020	951			
tRNA[Val]	H	1021	1092	72			
16S rRNA	H	1093	2767	1675			
tRNA[Leu (UAA)]	H	2768	2842	75			
ND1	H	2843	3817	975	ATG	TAG	2
tRNA[Ile]	H	3820	3889	70			1
tRNA[Gln]	L	3891	3962	72			
tRNA[Met]	H	3963	4031	69			
ND2	H	4032	5077	1046	ATG	TA-	
tRNA[Trp]	H	5078	5146	69			1
tRNA[Ala]	L	5148	5216	69			
tRNA[Asn]	L	5217	5290	74			
OL[d]	-	5291	5327	37			
tRNA[Cys]	L	5328	5394	67			1
tRNA[Tyr]	L	5396	5465	70			1
COI	H	5467	7023	1557	GTG	TAA	
tRNA[Ser (UGA)]	L	7024	7094	71			3
tRNA[Asp]	H	7098	7167	70			4
COII	H	7172	7862	691	ATG	T--	
tRNA[Lys]	H	7863	7936	74			1
ATP8	H	7938	8105	168	ATG	TAA	-10
ATP6	H	8096	8778	683	ATG	TA-	
COIII	H	8779	9564	786	ATG	TAA	2
tRNA[Gly]	H	9567	9636	70			
ND3	H	9637	9987	351	ATG	TAA	3
tRNA[Arg]	H	9991	10060	70			
ND4L	H	10061	10357	297	ATG	TAA	-7
ND4	H	10351	11731	1381	ATG	T--	
tRNA[His]	H	11732	11800	69			
tRNA[Ser (GCU)]	H	11801	11867	67			
tRNA[Leu(UAG)]	H	11868	11939	72			
ND5	H	11940	13772	1833	ATG	TAA	-4
ND6	L	13769	14290	522	ATG	TAG	
tRNA[Glu]	L	14291	14360	70			4
Cyt *b*	H	14365	15510	1146	ATG	TAA	1
tRNA[Thr]	H	15512	15584	73			2
tRNA[Pro]	L	15587	15655	69			
D-loop	-	15656	16730	1075			

[a] *H = heavy strand; L = light strand;* [b] *T or TA = incomplete stop codon;* [c] *Intergenic spacer = nucleotides gaps with negative numbers indicate overlapping;* [d] *OL = origin of light strand replication.*

Comparison of mitogenomes

To infer the genetic variability among mitogenomes, the *S. microcephalus* mitogenome was compared with the 17 related shark species mitogenomes and the two outgroups of Chimaeras and Batoids (Fig. 2). The mitogenome comparison analysis showed the same gene arrangement in all analyzed species. *S. pacificus* appeared as the most similar species to *S. microcephalus* with ≥ 98 % of nucleotide similarity. While the remaining Squaliformes species, *Squalus acanthias*, *S. montalbani* and *Cirrhigaleus australis* showed ≥ 88 % nucleotide similarity. The rest of the mitogenomes showed less nucleotide similarity to *S. microcephalus*, although in some genes, such as tRNA^{Phe} or 12S rRNA, ≥ 88 % nucleotide similarity was observed. The most diverged mitogenomic region among the species was the control region where the *S. microcephalus* control region differed (0 - 100 %) from the other species. Regarding the species from the two outgroups, high percentage of nucleotide similarity to *S. microcephalus* (88 - 92 %) were shown in small parts of their mitogenomes (e.g. at the end of 16S rRNA gene).

To evaluate the nucleotide similarities between the selected species, a pairwise was done comparing all the mitogenomes. The results showed a gradient of nucleotide similarity from black (high similarity) to white (less similarity) gradient colors (Appendix V). The results showed more than 75 % nucleotide similarity between all shark species, while the species from the outgroups had 70 % nucleotide similarity or less compared to the rest of the sharks. Among the shark species, *Pristiophorus japonicus* and *Hexanchus nakamurai* were the exceptions, since their comparison with the rest of the species were less than 75 % nucleotide similarity. As expected, high percentage of nucleotide similarity was observed between species from the same order. The comparison between *S. microcephalus* and *S. pacificus* showed 99 % nucleotide similarity (Appendix V). Due to the high percentage of similarity, the genetic distance between both species was calculated. The nucleotide differences between both species was low, 0.97 % base substitutions per site (in other words, 99.03 % of similarity).
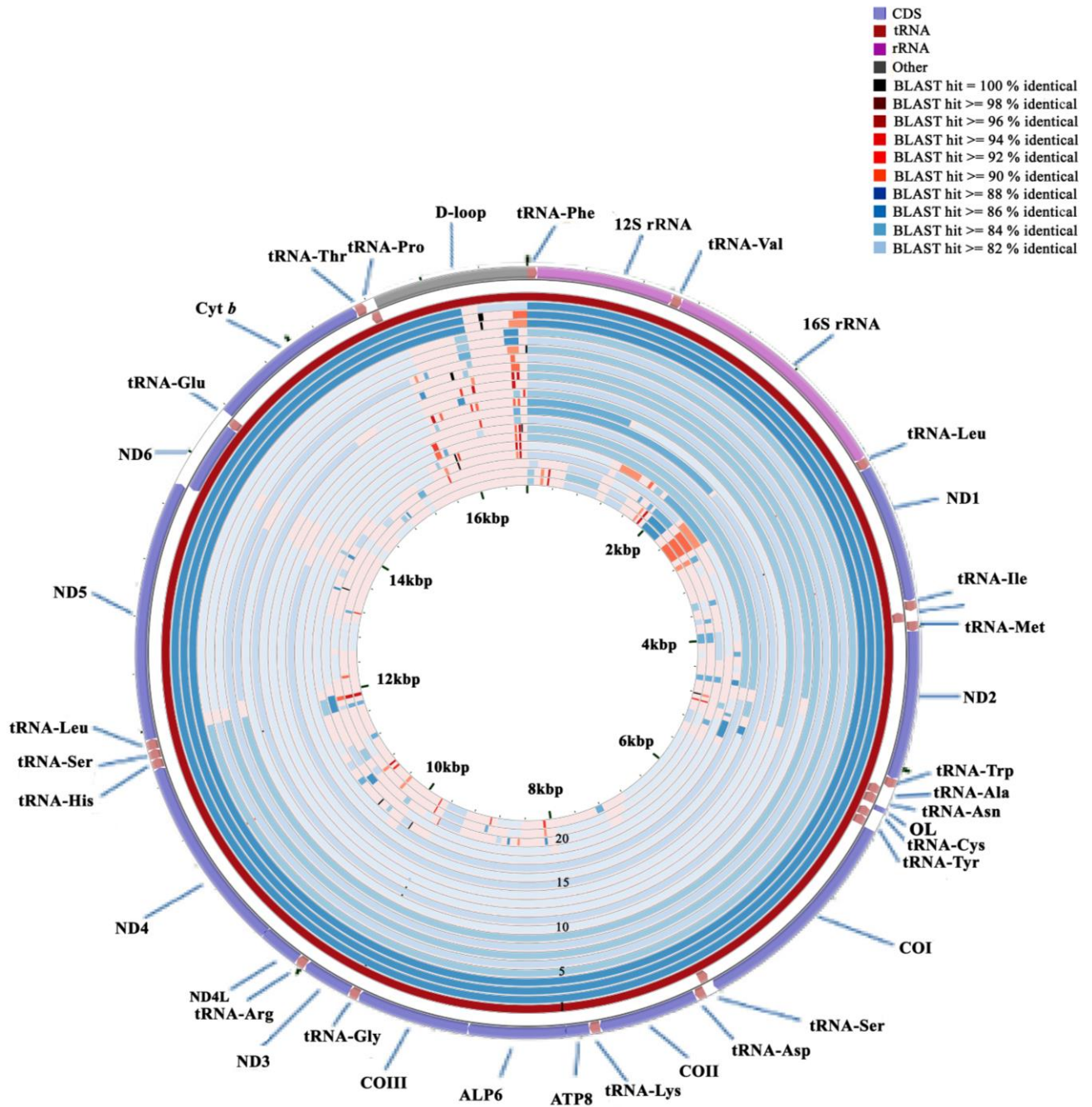
Figure 2 Graphical representation of the BLAST results generated by CGView comparison tool (CCT), showing the comparison of nucleotide similarity (%) between *S. microchepalus* reference mitogenome and 18 other shark species listed in Table 4. BLAST hits are displayed using different colors, depending on the degree of nucleotide similarities. The rings are enumerated from outside to the inside based on decreasing resemblance to *S. microcephalus*. First external mitogenome in dark red corresponds to *S. pacificus*, followed by *Squalus acanthias*, *Squalus montalbani*, *Cirrhigaleus australis*, *Squatina formosa*, *Squatina nebulosa*, *Squatina japonica*, *Heterodontus francisci*, *Pristiophorus japonicus*, *Alopias pelagicus*, *Notorynchus cepedianus*, *Hexanchus griseus*, *Pseudocarcharias kamoharai*, *Heterodontus zebra*, *Hexanchus nakamurai*, *Carcharias taurus*, *Alopias superciliosus*, and as outgroup, *Mobula japonica*, *Amblyraja radiata*, *Chimaera monstrosa*, *Callorhinchus milii* and *Callorhinchus callorynchus*.

Phylogenetic relationships

The phylogenetic position of *S. microcephalus* was investigated by phylogenetic reconstruction with 17 other shark species having available mitogenomes, and using Rays (Batoidea) and Chimaeras (Holocephali) as outgroup (Fig. 3). The Bayesian Inference tree showed the evolution of lineages between the analyzed species. Selachimorpha appeared as the sister group of Batoidea, having both, the Chimaeras as a common ancestor. Selachimorpha was divided in two major clades, Galeomorphii and Squalomorphii. Galeomorphii contained Heterodontiformes and Lamniformes orders, while Squalomorphii was composed by the rest of the analyzed orders; Hexanchiformes, Squatiniformes, Pristiophoriformes and Squaliformes (Fig. 3). All family-level relationships were monophyletic with two exceptions. Within the Lamniformes order, the family Alopiidae appeared paraphyletic with the Pseudocarchriidae and Odontaspidida (*Carcharias taurus*) families. Within the Squaliformes order, Somniosidae was paraphyletic with Squalidae. As expected, *S. microcephalus* together with *S. pacificus* compounded a sister group within the Squalidae family (*Squalus* and *Cirrhigaleus* genera) (Fig. 3). These two families (Somniosidae and Squalidae) from the Squaliformes order, had *Pristiophorus japonica* species as ancestor. The Squatiniformes order appeared as the sister group of the clade comprised by Pristiophoriformes and Squaliformes (Fig. 3).

The phylogenetic relationships among sharks were well resolved with high posterior probabilities (100 %) on the branches (Fig. 3). The exceptions occurred in the division of Squatinifomes with Pristiophoriformes and Squaliformes, and between *Alopias* and *Pseudocarcharias* genera from the Lamniformes order; with 98 % and 61 % of posterior probability respectively (Fig. 3). When exploring the phylogenetic position of *S. microcephalus* using ML and NJ trees, similar topologies were observed as for the Bayesian inference tree (Appendix VI).
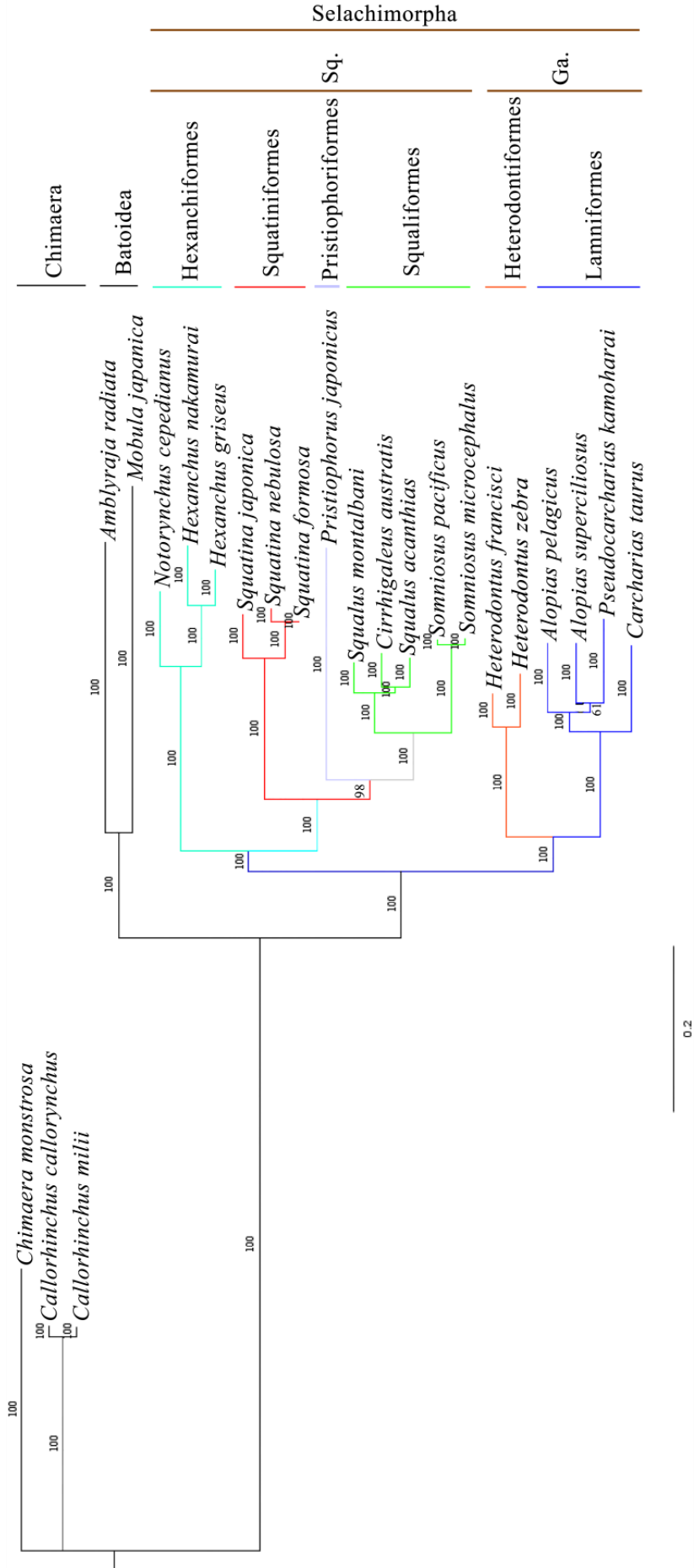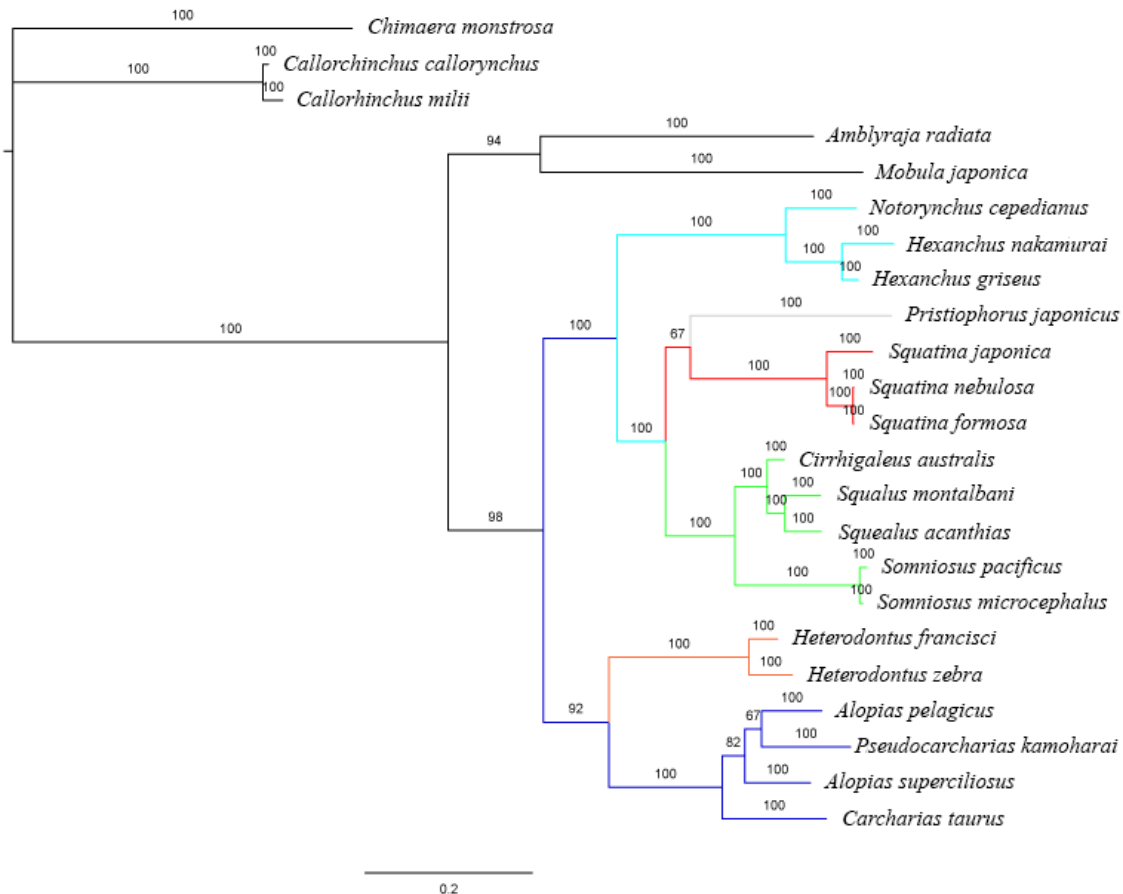
Figure 3 The Bayesian Inference tree colored by orders, outgroup species in black. Represented in brown, Squalomorphii (Sq.) and Galeomorphii (Ga.) clades that comprised the Selachimorpha superorder. The numbers on the branches indicate posterior probabilities in percentage. Branch length is proportional to the amount of genetic changes (nucleotide substitutions per site).

The influence of using single genes versus mitogenomes to infer phylogenetic positions, was evaluated by making phylogenetic reconstructions of all selected shark species (Chimaeras and Batoids as outgroups) using the COI gene and the control region (Fig. 4). The Bayesian Inference tree using the COI gene, showed similar topology as the mitogenome (Fig. 4A). The exception was found in the position of *Pristiophorus japonicus*, which was placed as sister group of the Squatiniformes (Fig. 4A). In contrast, when performing the phylogenetic analysis using the control region, the topology from the Bayesian Inference tree, differed from what was observed when the mitogenomes were used to place each of species (Fig. 4B). The clade composed by the Squatiniformes, Squaliformes and Pristiophorifores orders, was placed as sister group of the clade composed by Batoids, Lamniformes, Heterodontiformes and Hexanchiformes orders. Moreover, *Mobula japonica* appeared as sister group of Galeomorphii shark species and not as their ancestor together with the other Batoid, *Amblyraja radiata*. *Pristiophorus japonicus* was placed as sister group of the Squatiniformes when both, the COI gene and the control region, were used. Thus, the COI gene and the control region Bayesian inference trees showed different evolutionary patterns of analyzed species.
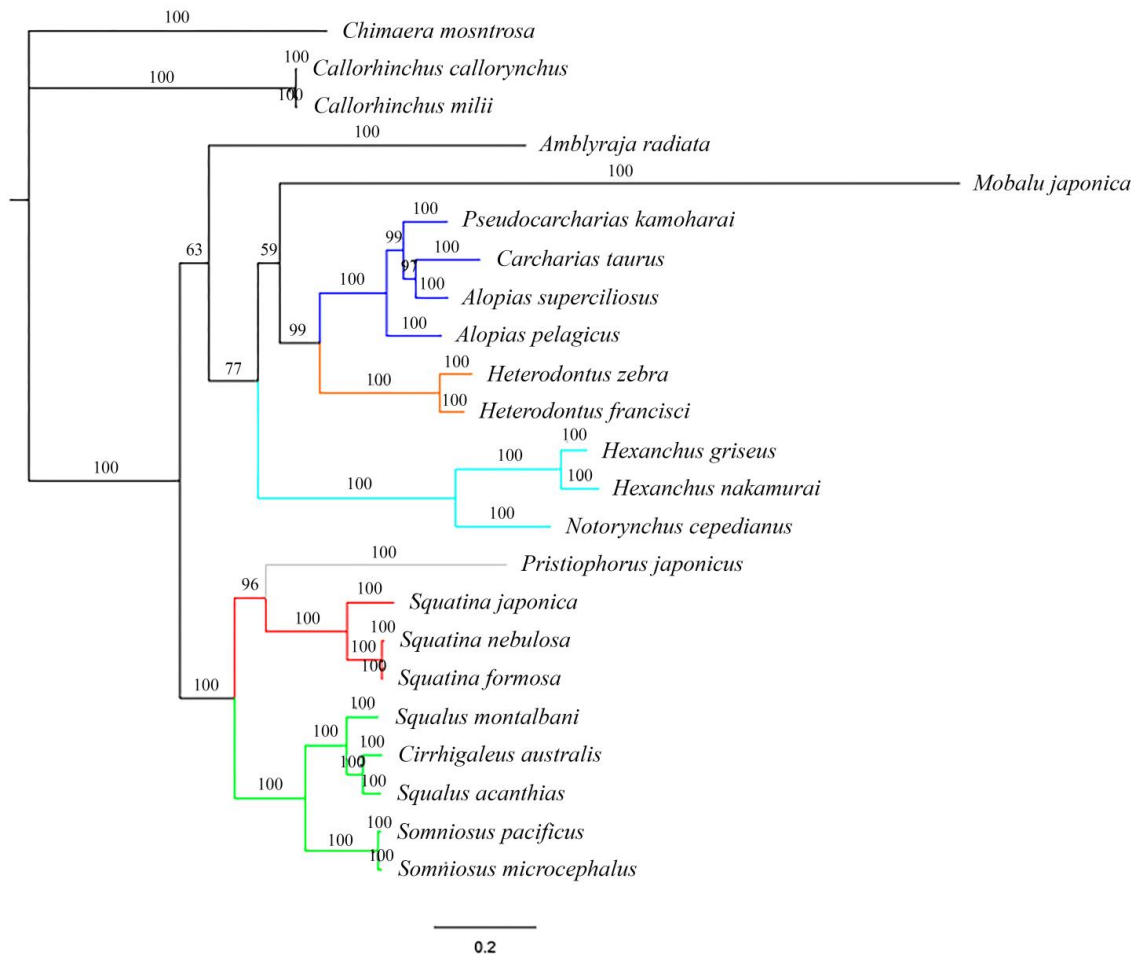
A

B

Figure 4. Bayesian inference tree of COI (A) and control region (B) colored by orders, outgroup species in black. The numbers on the branches indicate posterior probabilities in percentage. Branch length is proportional to the amount of genetic changes (nucleotide substitutions per site).

Divergence time estimates

The divergence time estimates tree, using the closing of the Isthmus of Panama, showed that sharks diverged from Batoids 238.28 Mya in the Triassic period (Fig. 5). The two main clades, Galeomorphii and Squalimorphii, diverged in the beginning of Lower Jurassic, 190 Mya. During the Jurassic period all the orders of shark appeared (~200 - 145 Mya). The families appeared more recently (~100 - 33 Mya), during the Upper Cretaceous and Eocene. Specifically, the split between Somniosidae family and the Squalidae family took place 109.57 Mya, in the Lower Cretaceous. The most recent divergence time estimation was 8000 years ago, when *Squatina formosa* diverged from *Squatina nebulosa*. The *S. microcephalus* and *S. pacificus* diverged 3.5 Mya, at the end of Tertiary period (Fig. 5). Similar results were obtained when the initial opening of the Bering strait, was used at *Somniosus* sp. divergence point (Appendix VII). Since not all analyzed species inhabit the Arctic, the Isthmus of Panama approach was chosen to represent the molecular clock of this study (Fig. 5).
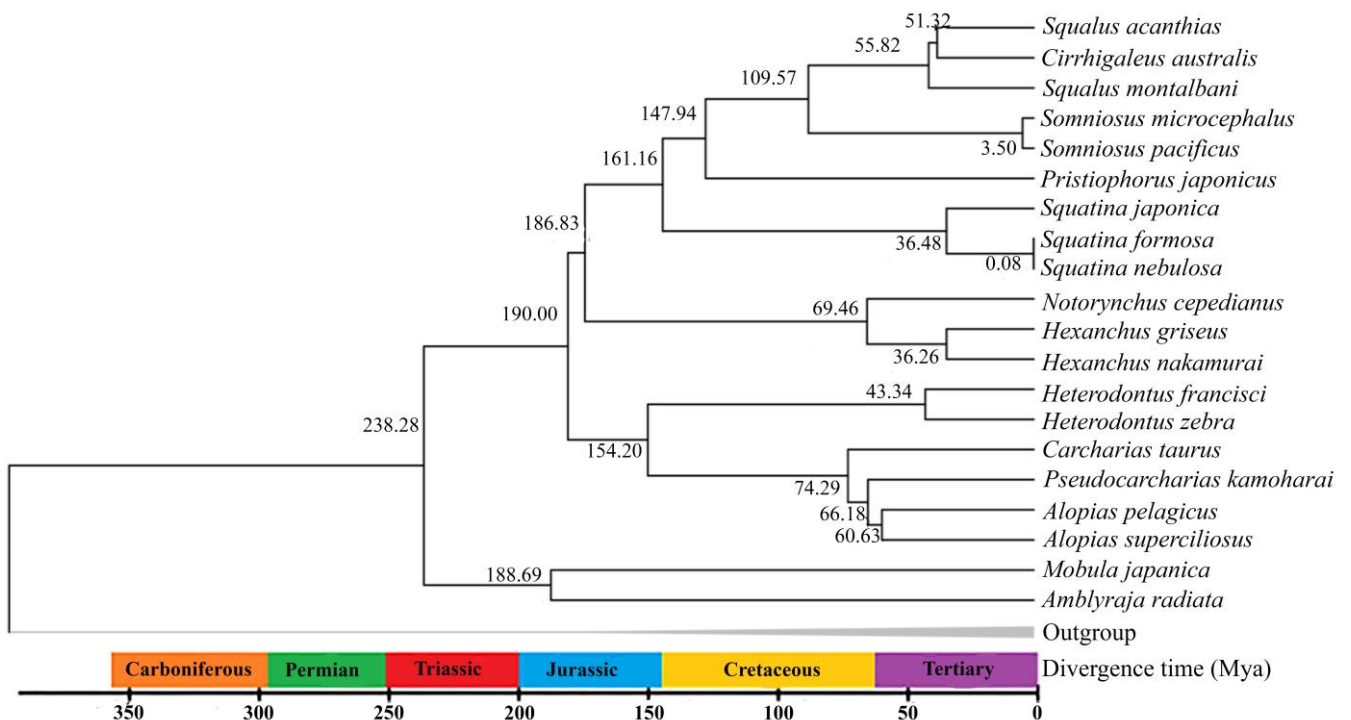

Figure 5 Time-tree inferred by ML analyses shows estimated divergence times of analyzed species. The outgroup appears as a grey line comprising the three species of Chimaeras (*Callorhinchus callorynchus*, *Callorhinchus milii*, *Chimaera monstrosa*). Branch lengths are proportional to divergence times measured by million years ago (Mya). The time scale and the divisions of geologic periods are indicated in different colors.

28

Discussion

This study provides an important investigation, for understanding more about the biology of the oldest living vertebrate known - *S. microcephalus*. The *S. microcephalus* mitogenome, with the typical structure of a vertebrate mitogenome, was placed as a sister species of *S. pacificus* on the phylogenetic tree of sharks. The results further revealed a high percentage (99 %) of nucleotide similarity between these two sleeper shark species. Moreover, it was confirmed that *S. microcephalus* and *S. pacificus* diverged 3.5 Mya, at the end of the Tertiary period.

The sequence and characterization of the *S. microcephalus* mitogenome was presented for the first time in this study. The size of the mitogenome was estimated to 16,730 bp composed by 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes and a control region. As hypothesized, the S. microcephalus mitogenome structure confirmed to be similar to other elasmobranchs (Alam et al., 2014; Chai et al., 2014; Chang et al., 2014a; Yang et al., 2016). Similar nucleotide composition, number and arrangement of the genes have been documented in multiple other vertebrates' mitogenomes (Brown, 1985; Miya & Nishida, 2015). At nucleotide level, AT content (60.6 %) was higher than GC content (39.4 %) in *S. microcephalus*, similar to other sharks and teleosts (Miya et al., 2003; Alam et al., 2014; Chang et al., 2014a). The incomplete stop codons found in the *S. microcephalus* mitogenome, are common among mitochondrial genes from vertebrates (Hou et al., 2007; Ki et al., 2010). The incomplete stop codons may be completed as TAA by posttranscriptional polyadensylation (addition of adenine monophosphates) of the 3´end that produce the mRNA maturation for translation (Anderson et al., 1981; Ojala et al., 1981). It has been suggested that the incomplete stop codons appeared as a selective pressure, to reduce the genome size by losing the unnecessary genes (Rand, 1993; Selosse et al., 2001). Moreover, it should be mentioned, that some genes could complete their stopped codons within the overlapping portion of the next genes. The incomplete stop codons (TA or T) obtained in the *S. microcephalus* mitogenome for ND2, COII, ATP6, ND4 protein-coding genes, were also observed in other sharks such us, *Rhincodon typus* (Whale shark, Alam et al. 2014), *Pseudocarcharias kamoharai* (Crocodile shark, Chang et al., 2014b) *Cetorhinus maximus* (Basking shark, Hester et al., 2015) and *Carcharhinus acronotus* (Black nose shark, Yang et al., 2016).

Comparison of mitogenomes

The comparison of the *S. microcephalus* mitogenome with the other shark species, indicated high nucleotide similarities varying between 81 and 99 % (Fig. 4). Not surprisingly, *S. pacificus* was found as the most similar species to *S. microcephalus*, suggesting that both were closely related species and had a recent common ancestor (Tamura et al., 2013). The second most similar species to *S. microcephalus* was *Squalus acanthias*. The wide distribution of *S. acanthias* in the Atlantic and Pacific Oceans, might have led to share genes with *S. microcephalus* in the past (Burgess, 2002; Compagno et al., 2005; Veríssimo et al., 2010). The high variability of the control region in the mitogenomes of analyzed species, has been also shown in other studies (Castro et al., 2007; Alam et al., 2014; Díaz-Jaimes et al., 2016). In contrast, the less variable (or more conserved) region from the mitogenome comparison was the COI, a protein-coding gene commonly used for DNA barcoding in species identification (Hebert et al., 2003; Ward et al., 2009; Lynghammar et al., 2014).

The comparison between all shark mitogenomes showed that the two sharks with the longest mitogenomes, *Pristiophorus japonicus* (18,430 bp) and *Hexanchus nakamurai* (18,650 bp) had the lowest percentage of nucleotide similarity compared to the rest (Appendix V). The different sizes between mitogenomes, corresponded to the high amount of tandem repeats in the control region of elasmobranchs (Macey et al., 1997; Castro et al., 2007).

Phylogenetic analysis

The phylogenetic position of *S. microcephalus* was inferred based on its mitogenome. The Bayesian, ML and NJ trees showed consistent results for the evolutionary relationships among the analyzed species (Fig. 2 and Appendix VI). Batoids and sharks appeared as sister group, as it has also been observed in other studies (Compagno, 1973; Douady et al., 2003; Naylor et al., 2005; Vélez-Zuazo & Agnarsson, 2011). Conversely, Shirai (1992, 1996) based on morphological traits, suggested that Batoids have been derived from sharks and not from the Chimaeras. The Bayesian tree clearly indicated a division of Selachimorpha in two clades, Squalomorphii and Galeomorphii, which also agrees with previous studies using single mitochondrial and nuclear genes (Naylor et al., 2005; Heinicke et al., 2009; Vélez-Zuazo & Agnarsson, 2011). The position of *S. microcephalus* was consistent with the traditional phylogeny tree of sharks based on a few mitochondrial genes (Vélez-Zuazo & Agnarsson,

2011). Furthermore, the location of *S. microcephalus* was also consistent with previous studies, where a single gene (Cyt *b*) was used (Sorenson et al., 2014; Straube et al., 2015).

Vélez-Zuazo & Agnarsson (2011) used single genes, four mitochondrial genes (Cyt *b*, COI, NADH2 and 16 rRNA) and one nuclear gene (Rag-1), to infer the phylogenetic reconstruction of all Selachimorpha (Elasmobranchs, Chondricthyes). They placed *Squatina nebulosa* as a sister group of all other Selachimorpha shark species, while in this study, using mitogenomes, *Squatina nebulosa* appeared within the order Squatiniformes, together with the others *Squatina* sp. Vélez-Zuazo & Agnarsson (2011) hypothesized that the singular position of the *Squatina nebulosa* could have been due to missing data. They also questioned the position of *Pristiophorus japonicus*, since was placed as the sister group of the Squaliformes order and not as the sister group of the Squatiniformes order with the rest of species from *Pristiophorus* genus. They argued that the position of *Pristiophorus japonicus* could have been due to missing data. However, the results based on mitogenomic inference, obtained herein, confirmed the position of *Pristiophorus japonicus* in the phylogeny.

The use of mitogenomes and single genes have been shown to influence the phylogenetic inference (Duchêne et al., 2011). When the COI gene was analyzed in this study, a similar topology of the tree, as when based on the mitogenome analysis, was obtained (Fig. 4A), while the control region, showed a different topology (Fig. 4B). The differences in the evolutionary patterns of both genes, confirmed on one hand, the conservative feature of the COI region (Brown, 1985) and on the other hand, the highly divergence characteristic of the control region (Avise et al., 1987). The consistent results that could be inferred from the analysis made by the control region, was that the species were grouped by orders (except for Batoids). Duchêne et al. (2011), observed that the use of single genes can provided incongruence topologies and less precise date estimates, although adding more genes, even the mitogenome, can improve the results. Furthermore, Jacobsen et al. (2012) observed that the use of the mitogenome improved the divergence times in recent diverged species. The COI gene and the control region are often used in DNA barcoding for species identifications (Hebert et al., 2003; Ward et al., 2005; Ward et al., 2008; Ward et al., 2009; Lynghammar et al., 2014) and in population genetic studies (Avise et al., 1987; Harrison, 1989; Castro et al., 2007) respectively. Thus, the strength of using mitogenomes instead single genes, was clear from the results obtained in the phylogenetic analysis that support the position of the *S. microcephalus*.

Divergence time estimates

The earliest fossil records suggested that many of the modern elasmobranchs (Neoselachii) groups, already diverged in the Lower Jurassic (199 - 175 Mya), belonged to the Tethys realm (Maisey, 2012). The earliest teeth were attributable to the Lower Triassic of Turkey (Maisey, 2012). Somniosids together with Squalids, Squatiniforms, Lamniforms and the rest of analyzed species in this study, except for the Chimaeras, appeared during Jurassic and Cretaceous periods (199 - 65 Mya) and belonged to Tethys realm (Maisey, 2012). The break-up of the Gondwana continent, 200 Ma, followed by the opening of the North Atlantic Ocean in the Triassic and the South Atlantic in the Cretaceous, led to a diversification and expansion of elasmobranchs from the European and the Caribbean Tethys into the new oceans (Maisey, 2012). Most of the shark have been suggested to appear during the Jurassic period (~200 - 145 Mya), while most of the families have been estimated to originate to the Upper Cretaceous and Eocene periods (~100 - 33 Mya), supporting the data based on elasmobranchs fossil records (Underwood, 2006). The mitogenomic results herein, indicated that Batoids and sharks diverged approximately 238 Mya (Fig. 5) in the Triassic period, which disagrees with 364 Mya estimated by Sorenson et al. (2014). However, the estimation obtained by Kriwet et al. (2009) (~197 Mya), was closer to the results herein. Nevertheless, the studies of Sorenson et al. (2014) and Kriwet et al. (2009) used single genes, which may suggest that the divergence time estimates obtained herein are more accurate as mitogenomic inference have been shown to be more informative in phylogenetic studies (Jacobsen et al., 2012).

The reason for using two different geological events approaches to infer when *S. microcephalus* was diverged from *S. pacificus*, was due to the connection via the Bering strait and the Isthmus of Panama of *S. microcephalus* and *S. pacificus* in the geological past. The Bering strait connects North Pacific and Arctic Oceans, while the Isthmus of Panama connects the West Atlantic and East Pacific Oceans. The difference in the divergence time estimates was 3.5 Mya for the Isthmus of Panama (Fig. 5) and 4.87 Mya for the Bering strait (Appendix VII). The divergence between *S. microcephalus* and *S. pacificus* was correlated with the Isthumus of Panama geological event as hypothesized. Murray et al. (2008) also obtained 3.5 Mya as the divergence time, based on Cyt *b* mitochondrial gene and the Isthmus of Panama geological event. Thus, this study confirmed the divergence of *S. microcephalus* and *S. pacificus* at 3.5 Mya. The Isthmus of Panama geological event was chosen to represent the time-tree in this study, since most of the analyzed sharks have not been recorded in the Arctic Ocean. Although, the divergence time estimates inferred when considering the Bering strait event, should be taken

into consideration for further investigations. The divergence estimation between *S. microcephalus* and *S. pacificus*, showed a recent divergence during Pliocene epoch (5.3 - 1.8 Mya), when compared with the rest of estimations (Fig. 5).

S. microcephalus - *S. pacificus* **speciation**

In the middle of the Pliocene, the oceans started to cool due to the closing of the Isthmus of Panama around 3.1 and 3.5 Mya (Coates & Obando, 1996; Coates et al., 2004). Thus, the Atlantic Ocean was not receiving warm water from the Pacific Ocean anymore (Bartoli et al., 2005). This event may suggest a distribution of *Somniosus sp.* towards the North Atlantic Ocean defining two different populations, todays *S. microcephalus* and *S. pacificus*. The first opening of the Bering strait was earlier, 4.8 - 5.5 Mya (Gladenkov et al., 2002). By that time, the oceans of the world were warm, 2.5 - 3 ºC higher than today (IPPC, 2007). A few million years after the opening of the Bering strait, a glaciation occurred (2.5 Mya) (Schaefer, 1953). Thus, the *Somniosus* sp. from the Pacific Ocean, could have been swimming through the Bering strait to the North Atlantic Ocean, resulting on a division of the *Somniosus* sp. in two different populations. This may have been driven by allopatric speciation where *S. microcephalus* and *S. pacificus* physically have been divided and subsequent adaptation towards the environment and ecological niches have driven the divergence (concept discussed in Schluter, 2000). However, based on the mitogenomic data there was still less than 1 % nucleotide difference between these closely related species, making it an open question whether they actually can be considered to be two different species. Ward et al. (2009) suggested 3.5 % of genetic distance as a universal gap value for fishes in order to consider them as two different species based on DNA barcoding. DNA barcoding cannot always solve the problem of species distinction (Trewick, 2008), therefore this study tried to obtain more information about the division of *S. microcephalus* and *S. pacificus*, based on their mitogenomes.

Previous studies have investigated the genetic diversity of the *Somniosus* sp. Murray et al. (2008) and the *S. microcephalus* Hussey et al. (2014), based on the Cyt *b* mitochondrial gene. Murray et al. (2008) found *S. microcephalus* and *S. pacificus* to be two distinct species, and *S. pacificus* and *S. antarcticus* to be the same species. *S. antarcticus*, also a species from *Somniosus* subgenus, lives in the Southern hemisphere (Yano et al., 2004). Taking into account the distribution of *S. antarcticus* and the suggestion of *S. pacificus* and *S. antarcticus* to be a single species (Murray et al., 2008), suggested that *S. pacificus* was not restricted to the north

Pacific Ocean. Murray et al. (2008) indicated that the gene flow between *S. microcephalus* and *S. pacificus* was negligible based on the maximum likelihood estimations of migration between the two species. However, Hussey et al. (2014) found two juvenile *S. microcephalus* carrying a *S. pacificus* mitochondrial Cyt *b* haplotype (H4). This finding showed possible hybridization/gene flow at some point between the species, not confirming the estimates made by Murray et al. (2008). Thus, taking the result of Hussey et al. (2014) into account, the low genetic distance obtained between the mitogenomes of *S. microcephalus* and *S. pacificus* observed herein, together with the similar body morphologies of the two species (Bigelow & Schroeder, 1944; Yano et al., 2004; MacNeil et al., 2012), diet and life histories (Yano et al., 2007), support the possibility of a continuous distribution of *Somniosus* subgenus across the globe, being a single species. Thus, the mitogenomic data did not support the hypothesis that *S. microcephalus* and *S. pacificus* were two different species. Although the increase of sampling for each of the species would improve the estimates on whether the gene flow had occurred among the three sleeper sharks or not. Nuclear DNA estimates would give more information about the migration rates and population divergences. Notwithstanding this, *S. microcephalus* and *S. pacificus* may be at the early stages of speciation, but the recent genomic divergence (3.5 Mya) and the events of gene flow (Hussey et al., 2014) may have contributed to an "artificial" shallow mitogenomic divergence. Hence, further mitogenomic investigations on phylogeography and population structures between and within the three sleeper sharks from the *Somniosus* subgenus are needed in order to establish the species status of *S. microcephalus*.

Concluding remarks

The size of the *S. microcephalus* reference mitogenome was estimated to 16,730 bp and was shown to consist of 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes, and a control region. The *S. microcephalus* mitogenome was, thus, similar to most other vertebrate mitogenomes.

The Bayesian inference, ML, and NJ trees topologies were similar based on mitogenomic data, confirming the position of *S. microcephalus* on the phylogenetic tree of sharks. *S. microcephalus* was positioned as a sister species of *S. pacificus*. In addition, *S. microcephalus* appeared, together with *S. pacificus*, as the most derived species, compared to the rest of analysed shark species. Single genes appeared to be less informative than mitogenome, as their analysis showed incongruent topologies in the phylogenetic trees. Divergence time estimates confirmed the recent divergence (3.5 Mya) of *S. microcephalus* and *S. pacificus*. The low genetic difference (0.97 %) between these closely related species and their similar morphologies, suggested to be the same species. Thus, the three sleeper shark species comprising the *Somniosus* subgenus (*S. microcephalus*, *S. pacificus* and *S. antarcticus*) are suggested to be a single species with a continuous distribution across the globe.

The mitogenomic resource obtained in the present study will form an important asset for the continued studies of the *S. microcephalus*. Especially, it will assist in future phylogeographical and population genetic studies, to elucidate the inter-species and intra-species genetic variation. Such studies would aid in establishing the species status of the *Somniosus* subgenus. The resource will also contribute to a more holistic conservation management of the species as it allows for establishing evolutionary significant units (subspecies). Moreover, the mitogenome will also be important for understanding the biology (e.g. metabolism and temperature adaptation) of this elusive species as the resource provide the needed tool for studying the responses of single genes under different environmental settings.

References

Alam, M. T., R. A. Petit, T. D. Read & A. D. M. Dove, 2014. The complete mitochondrial genome sequence of the world's largest fish, the whale shark (*Rhincodon typus*), and its comparison with those of related shark species. Gene 539 (1): 44-49.

Aljanabi, S. M. & I. Martinez, 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Research 25 (22): 4692-4693.

Anderson, S., A. T. Bankier, B. G. Barrell, M. H. L. De Bruijn, A. R. Coulson, J. Drouin, I. C. Eperon, D. P. Nierlich, B. A. Roe, F. Sanger, P. H. Schreier, A. J. H. Smith, R. Staden & I. G. Young, 1981. Sequence and organization of human mitochondrial genome. Nature 290: 457-465.

Arnason, U., A. Gullberg & A. Janke, 2001. Molecular phylogenetics of gnathostomous (jawed) fishes: Old bones, new cartilage. Zoologica Scripta 30 (4): 249-255.

Avise, J. C., J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. E. Neigel & N. C. Saunders, 1987. Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. Annual Review of Ecology and Systematics 18 (1): 489-522.

Barril, P. & S. Nates, 2012. Introduction to agarose and polyacrylamide gel electrophoresis matrices with respect to their detection sensitivities. Gel Electrophoresis - Principles and Basics: 3-15.

Bartoli, G., M. Sarnthein, M. Weinelt, H. Erlenkeuse, D. Garbe-Schönberg & D. W. Lea, 2005. Final closure of Panama and the onset of northern hemisphere glaciation. Earth and Planetary Science Letters 237: 33-44.

Bigelow, H. B. & W. C. Schroeder, 1944. New sharks from the western north Atlantic. Proceedings of the New England Zoological Club 23: 21-36.

Bigelow, H. B. & W. C. Schroeder, 1948. Sharks. Fishes of the western North Atlantic. Part One. Lancelets, Cyclostomes, Sharks: 59-546.

Brown, W. M., 1985. The mitochondrial genome of animals. In: MacIntyre, R. J., (eds.) Molecular evolutionary genetics. Plenum Press, New York: 95-130.

Burgess, G. H., 2002. Spiny dogfishes—Family Squalidae. In: Colette, B. B. & G. Klein-MacPhee, (eds.) Bigelow and Schroeder's fishes of the Gulf of Maine. Smithsonian Institution Press, Washington, D. C.: 54-57.

Cappetta, H., 1993. Sélaciens nouveaux (Chondrichthyes, Neoselachii) du Paléocène supérieur de la région d'Ouarzazate, Maroc. Paläontologische Zeitschrift 67 (1-2): 109-122 (In

French).

Castro, A. L. F., B. S. Stewart, S. G. Wilson, R. E. Hueter, M. G. Meekan, P. J. Motta & S. A. Karl, 2007. Population genetic structure of Earth's largest fish, the whale shark (*Rhincodon typus*). Molecular Ecology 16 (24): 5183-5192.

Chai, A., A. Yamaguchi, K. Furumitsu & J. Zhang, 2014. Mitochondrial genome of Japanese angel shark *Squatina japonica* (Chondrichthyes: Squatinidae). Mitochondrial DNA 1736: 1-2.

Chang, C. H., K. T. Shao, Y. S. Lin, H. C. Ho & Y. C. Liao, 2013. The complete mitochondrial genome of the big-eye thresher shark, *Alopias superciliosus* (Chondrichthyes, Alopiidae). Mitochondrial DNA 1736: 1-3.

Chang, C. H., R. W. Jabado, Y. S. Lin & K. T. Shao, 2014a. The complete mitochondrial genome of the sand tiger shark, *Carcharias taurus* (Chondrichthyes, Odontaspididae). Mitochondrial DNA 1736: 1-2.

Chang, C. H., K. T. Shao, Y. S. Lin & H. C. Ho, 2014b. The complete mitochondrial genome of the crocodile shark, *Pseudocarcharias kamoharai* (Chondrichthyes, Lamnidae) Mitochondrial DNA, In press: 1-2.

Chen, X., D. Xiang, W. Ai & X. Shi, 2013. Complete mitochondrial genome of the pelagic thresher *Alopias pelagicus* (Lamniformes: Alopiidae). Mitochondrial DNA 1736: 1-2.

Chen, X., X. Peng, X. Huang & D. Xiang, 2014. Complete mitochondrial genome of the Zebra bullhead shark *Heterodontus zebra* (Heterodontiformes: Heterodontidae). Mitochondrial DNA 25 (4): 280-281.

Coates, A. G. & J. A. Obando, 1996. The geologic evolution of the Central American Isthmus. In: Jackson, J. B. C., A. F. Budd & A. G. Coates, (eds.) Evolution and environment in tropical America. University of Chicago Press, Chicago: 21-56.

Coates, A. G., L. S. Collins, M. P. Aubry & W. A. Berggren, 2004. The geology of the Darien, Panama, and the late Miocene-Pliocene collision of the Panama arc with northwestern South America. Geological Society of America Bulletin 116: 1327-1344.

Compagno, L. J. V., 1973. Interrelationships of living elasmobranch. Zoological Journal of the Linnean Society 53: 15-61.

Compagno, L. J. V., M. Dando & S. Fowler, 2005. Sharks of the World. Princeton University Press, Princeton, NJ.

Corrigan, S., L. Yang, P. J. Cosmann & G. J. Naylor, 2014. A description of the mitogenome of the Endangered Taiwanese angelshark, *Squatina formosa*. Mitochondrial DNA, In press: 1-2.

Curole, J. P. & T. D. Kocher, 1999. Mitogenomics: Digging deeper with complete mitochondrial genomes. Trends in Ecology and Evolution 14 (10): 394-398.

Díaz-Jaimes, P., N. J. Bayona-Vásquez, D. H. Adams & M. Uribe-Alcocer, 2016. Complete mitochondrial DNA genome of bonnethead shark, *Sphyrna tiburo*, and phylogenetic relationships among main superorders of modern elasmobranchs. Meta Gene 7: 48-55.

Darriba, D., G. L. Taboada, R. Doallo & D. Posada, 2012. jModelTest 2: More models, new heuristics and parallel computing. Nature Methods 9 (8): 772-772.

Deep-C., 2013. Deep-C scientists capture first Greenland shark in the Gulf of Mexico. Retrieved from: https://deep-c.org/news-and-multimedia/in-the-news/deep-c-scientists-capture-first-greenland-shark-in-the-gulf-of-mexico.

Douady, C. J., M. Dosay, M. S. Shivji & M. J. Stanhope, 2003. Molecular phylogenetic evidence refuting the hypothesis of Batoidea (rays and skates) as derived sharks. Molecular Phylogenetics and Evolution 26 (2): 215-221.

Drummond, A. J. & A. Rambaut, 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7: 214.

Duchêne, S., F. I. Archer, J. Vilstrup, S. Caballero & P. A. Morin, 2011. Mitogenome phylogenetics: The impact of using single regions and partitioning schemes on topology, substitution rate and divergence time estimation. PLoS ONE 6 (11): 1-13.

Dunbar, M. J. & H. H. Hildebrand, 1952. Contribution to the study of fishes of Ungava Bay. Journal of the Fisheries Research Board of Canada 9: 83-128.

Galtier, N., B. Nabholz, S. Glémin & G. D. D. Hurst, 2009. Mitochondrial DNA as a marker of molecular diversity: A reappraisal. Molecular Ecology 18 (22): 4541-4550.

Gao, Y., T. Liu, T. Wei, X. Geng, J. Wang & H. Ma, 2014. Complete mitochondrial genome of the Clouded angelshark (*Squatina nebulosa*). Mitochondrial DNA 27 (3): 1599-1600.

Gilbert, M. T. P., D. I. Drautz, A. M. Lesk, S. Y. W. Ho, J. Qi, A. Ratan & S. C. Schuster, 2008. Intraspecific phylogenetic analysis of Siberian woolly mammoths using complete mitochondrial genomes. Proceedings of the National Academy of Sciences 105 (24): 8327-32.

Gladenkov, A. Y., A. E. Oleinik, L. Marincovich & K. B. Barinov, 2002. A refined age for the earliest opening of Bering Strait. Palaeogeography, Palaeoclimatology, Palaeoecology 183 (3-4): 321-328.

Grant, J. R., A. S. Arantes & P. Stothard, 2012. Comparing thousands of circular genomes using the CGView Comparison Tool. BMC Genomics 13 (1): 202.

Guindon, S. & O. Gascuel, 2003. A simple, fast, and accurate method to estimate large

phylogenies by maximum likelihood. Systematic Biology 52 (5): 696-704.

Harrison, R. G., 1989. Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. Trends in Ecology and Evolution 4 (1): 6-11.

Hebert, P. D., S. Ratnasingham & J. R. deWaard, 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society of London. Series B, Biological Sciences 270: 96-99.

Heinicke, M. P., G. J. P. Naylor & S. B. Hedges, 2009. Cartilaginous fishes (Chondrichthyes). The Timetree of Life 9: 320-327.

Hester, J., K. Atwater, A. Bernard, M. Francis & M. S. Shivji, 2015. The complete mitochondrial genome of the basking shark *Cetorhinus maximus* (Chondrichthyes, Cetorhinidae). Mitochondrial DNA 26 (5): 730-1.

Hobson, K. A., A. Fisk, N. Karnovsky, M. Holst, J. M. Gagnon & M. Fortier, 2002. A stable isotope (d13C, d15N) model for the North Water food web: Implications for evaluating trophodynamics and the flow of energy and contaminants. Deep-Sea Research Part II: Topical Studies in Oceanography 49 (22-23): 5131-5150.

Hou, W. R., Y. Chen, X. Wu, J. C. Hu, Z. S. Peng, J. Yang, Z. X. Tang, C. Q. Zhou, Y. M. Li, S. K. Yang, Y. J. Du, L. L. Kong, Z. L. Ren, H. Y. Zhang & S. R. Shuai, 2007. A complete mitochondrial genome sequence of Asian black bear Sichuan subspecies (*Ursus thibetanus mupinensis*). International Journal of Biological Sciences 3 (2): 85-90.

Hussey, N. E., A. Cosandey-Godin, R. P. Walter, K. J. Hedges, M. VanGerwen-Toyne, A. N. Barkley & A. T. Fisk, 2015. Juvenile Greenland sharks *Somniosus microcephalus* (Bloch & Schneider, 1801) in the Canadian Arctic. Polar Biology 38 (4): 493-504.

Inoue, J. G., M. Miya, K. Lam, B. H. Tay, J. A. Danks, J. Bell & B. Venkatesh, 2010. Evolutionary origin and phylogeny of the modern holocephalans (Chondrichthyes: Chimaeriformes): A mitogenomic perspective. Molecular Biology and Evolution 27 (11): 2576-2586.

Intergovernmental Panel on Climate Change (IPPC), 2007. The physical science basis— Contribution of working group I to the fourth assesment report of the Intergovernmental Panel on Clime Change, edited by S. Solomon et al., Cambridge Univerisity Press, New York: 996.

Iwasaki, W., T. Fukunaga, R. Isagozawa, K. Yamada, Y. Maeda, T. P. Satoh & M. Nishida, 2013. Mitofish and mitoannotator: A mitochondrial genome database of fish with an accurate and automatic annotation pipeline. Molecular Biology and Evolution 30 (11): 2531-2540.

Jacobsen, M. W., M. M. Hansen, L. Orlando, D. Bekkevold, L. Bernatchez, E. Willerslev & M. T. P. Gilbert, 2012. Mitogenome sequencing reveals shallow evolutionary histories and recent divergence time between morphologically and ecologically distinct European whitefish (*Coregonus* spp.). Molecular Ecology 21 (11): 2727-2742.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock & A. Drummond, 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28 (12): 1647-1649.

Kemper, J. M. & G. J. P. Naylor, 2015. The complete mitochondrial genome and phylogenetic position of the Philippines spurdog, *Squalus montalbani*. Mitochondrial DNA 1736: 1-2.

Ki, J. S., D. S. Hwang, T. J. Park, S. H. Han & J. S. Li, 2010. A comparative analysis of the complete mitochondrial genome of the Euroasian otter *Luttra lutra* (Carnivora; Mustelidae). Molecular Biology Reports 37 (4): 1943-55.

Klug, S. & J. Kriwet, 2010. Timing of deep-sea adaptation in dogfish sharks: Insights from a supertree of extinct and extant taxa. Zoologica Scripta 39 (4): 331-342.

Kriwet, J., W. Kiessling & S. Klug, 2009. Diversification trajectories and evolutionary life-history traits in early sharks and batoids. Proceedings of The Royal Society B 276 (1658): 945-951.

Kyne, P. M., S. A. Sherrill-Mix & G. H. Burgess, 2006. *Somniosus microcephalus*. The IUCN Red List of Threatened Species. Version 2015-4. <www.iucnredlist.org>. Downloaded on 8 October 2015.

Leclerc, L. M. E., C. Lydersen, T. Haug, L. Bachmann, A. T. Fisk & K. M. Kovacs, 2012. A missing piece in the Arctic food web puzzle? Stomach contents of Greenland sharks sampled in Svalbard, Norway. Polar Biology 35 (8): 1197-1208.

Lynghammar, A., J. S. Christiansen, A. M. Griffiths, S. E. Fevolden, H. Hop & T. Bakken, 2014. DNA barcoding of the northern Northeast Atlantic skates (Chondrichthyes, Rajiformes), with remarks on the widely distributed starry ray. Zoologica Scripta 43 (5): 485-495.

Macey, J. R., A. Larson, N. B. Ananjeva, Z. Fang & T. J. Papenfuss, 1997. Two novel gene orders and the role of light-strand replication in rearrangement of the vertebrate mitochondrial genome. Molecular Biology and Evolution 14: 91-104.

MacNeil, M. A., B. C. McMeans, N. E. Hussey, P. Vecsei, J. Svavarsson, K. M. Kovacs & A. T. Fisk, 2012. Biology of the Greenland shark *Somniosus microcephalus*. Journal of Fish Biology 80 (5): 991-1018.

Maisey, J. G., 2012. What is an "elasmobranch"? The impact of palaeontology in understanding

elasmobranch phylogeny and evolution. Journal of Fish Biology 80 (5): 918-951.

Martin, A. P., G. J. Naylor & S. R. Palumbi, 1992. Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. Nature 357 (6374): 153-155.

McMeans, B. C., J. Svavarsson, S. Dennard & A. T. Fisk, 2010. Diet and resource use among Greenland sharks (*Somniosus microcephalus*) and teleosts sampled in Icelandic waters, using δ13C, δ15N, and mercury. Canadian Journal of Fisheries and Aquatic Sciences 67: 1428-1438.

Miya, M., H. Takeshima, H. Endo, N. B. Ishiguro, J. G. Inoue, T. Mukai & M. Nishida, 2003. Major patterns of higher teleostean phylogenies: A new perspective based on 100 complete mitochondrial DNA sequences. Molecular Phylogenetics and Evolution 26 (1): 121-138.

Miya, M. & M. Nishida, 2015. The mitogenomic contributions to molecular phylogenetics and evolution of fishes: A 15-year retrospect. Ichthyological Research 62 (1): 29-71.

Moritz, C., 1994. Applications of mitochondrial DNA analysis in conservation: A critical review. Molecular Ecology 3 (4): 401-411.

Murray, B. W., J. Y. Wang, S. C. Yang, J. D. Stevens, A. Fisk & J. Svavarsson, 2008. Mitochondrial cytochrome b variation in sleeper sharks (Squaliformes: Somniosidae). Marine Biology 153 (6): 1015-1022.

Naylor, G. J. P., J. A. Ryburn, O. Fedrigo & A. Lopez, 2005. Phylogenetic relationships among the major lineages of modern elasmobranchs. In: Hamlett, W. C. & B. G. M. Jamieson, (eds.) Reproductive biology and phylogeny of Chondrichthyans (Sharks, skates, stingrays and chimaeras). University of Queensland Press 3: 1-25.

Nielsen, J., R. B. Hedeholm, M. Simon & J. F. Steffensen, 2014. Distribution and feeding ecology of the Greenland shark (*Somniosus microcephalus*) in Greenland waters. Polar Biology 37 (1): 37-46.

Nielsen et al., in press. Eye lens radiocarbon reveals centuries of longevity in the Greenland shark, *Somninosus microcephalus* (In press in Science).

Ojala, D., J. Montoya & G. Attardi, 1981. tRNA punctuation model of RNA processing in human mitochondria. Nature 290: 470-474.

Poortvliet, M. & G. Hoarau, 2013. The complete mitochondrial genome of the Spinetail Devilray, *Mobula japanica*. Mitochondrial DNA 24 (1): 28-30.

Quero, J. C., P. Decamps, G. Delmas, M. Duron & J. Fonteneau, 1982. Observations Ichthyologiques Effectuees en 1981. Annales de la societe des sciences naturelles de la Charente-Maritime. La Rochelle 6 (9): 1021-1028 (In French).

Rambaut, A., 2005-2016. FigTree v1.4.3. Institute of Evolutionary Biology, University of

Edinburgh, http://tree.bio.ed.ac.uk/.

Rand, D. M., 1993. Endotherms, ectotherms and mitochondrial genome-size variation. Journal of Molecular Evolution 37: 281-295.

Rasmussen, A. S. & U. Arnason, 1999. Phylogenetic studies of complete mitochondrial DNA molecules place cartilaginous fishes within the tree of bony fishes. Journal of Molecular Evolution 48 (1): 118-123.

Renz, A. J., A. Meyer & S. Kuraku, 2013. Revealing less derived nature of cartilaginous fish genomes with their evolutionary time scale inferred with nuclear genes. PLOS ONE 8 (6): 1-9.

Ronquist, F., M. Teslenko, P. Van Der Mark, D. L. Ayres, A. Darling, S. Höhna & J. P. Huelsenbeck, 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61 (3): 539-542.

Rozen, S. & H. Skaletsky, 2000. Primer3 on the WWW for general users and for biologist programmers. Methods in Molecular Biology 132: 365-386.

Selosse, M. A., B. Albert & B. Godelle, 2001. Reducing the genome size of organelles favors gene transfer to the nucleus. Trends in Ecology & Evolution 16: 135-141.

Schaefer, I., 1953. Die donaueiszeitlichen Ablagerungen an Lech und Wertach. Geologia Bavarica 19: 13-64 (In German).

Schluter, D., 2000. The Ecology of Adaptive Radiation. Oxford Series in Ecology and Evolution.

Shirai, S., 1992. Phylogenetic relationships of the angel sharks, with comments on elasmobranch phylogeny (Chondrichthyes, Squatinidae). Copeia 1992 (2): 505.

Shirai, S., 1996. Phylogenetic interrelationships of neoselachians (Chondrichthyes: Euselachii). In: Stassny, M. L. J., L. R. Parenti & G. D. Johnson, (eds.) Interrelationships of fishes, Academic Press, San Diego: 9-34.

Singh, T. R., G. Tsagkogeorga, F. F. Delsuc, S. Blanquart, N. Shenkar, Y. Loya & D. D. Huchon, 2009. Tunicate mitogenomics and phylogenetics: Peculiarities of the Herdmania momus mitochondrial genome and support for the new chordate phylogeny. BMC Genomics 10 (1): 534.

Sorenson, L., F. Santini & M. E. Alfaro, 2014. The effect of habitat on modern shark diversification. Journal of Evolutionary Biology 27 (8): 1536-1548.

Straube, N., C. Li, J. M. Claes, S. Corrigan & G. J. P. Naylor, 2015. Molecular phylogeny of Squaliformes and first occurrence of bioluminescence in sharks. BMC Evolutionary Biology 15 (1): 162.

Strohm, J. H. T., R. A. Gwiazdowski & R. Hanner, 2015. Mitogenome metadata: Current trends and proposed standards. Mitochondrial DNA, Early Online: 1-7.

Tamura, K., G. Stecher, D. Peterson, A. Filipski & S. Kumar, 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30 (12): 2725-2729.

Tanaka, K., T. Shiina, T. Tomita, S. Suzuki, K. Hosomichi, K. Sano & S. Tanaka, 2013. Evolutionary relations of hexanchiformes deep-sea sharks elucidated by whole mitochondrial genome sequences. BioMed Research International 2013: 11.

Tanaka, K., T. Shiina, T. Tomita, S. Kinjo, K. Hosomichi, K. Sano, H. Doi, A. Kono, T. Komiyama, H. Inoko, J. K. Kulski & S. Tanaka, Unpublished. Phylogeny of whole mitochondrial genomes from Hexanchiform sharks.

Templeman, W., 1963. Distribution of sharks in the Canadian Atlantic (with special reference to Newfoundland waters). Bulletin of the Fisheries Research Board of Canada 140: 36-42.

Trewick, S. A., 2008. DNA barcoding is not enough: Mismatch of taxonomy and genealogy in New Zealand grasshoppers (Orthoptera: Acrididae). Cladistics 24: 240-254.

Underwood, C. J., 2006. Diversification of the Neoselachii (Chondrichthyes) during the Jurassic and Cretaceous. Paleobiology 32 (2): 215-235.

Vélez-Zuazo, X. & I. Agnarsson, 2011. Shark tales: A molecular species-level phylogeny of sharks (Selachimorpha, Chondrichthyes). Molecular Phylogenetics and Evolution 58 (2): 207-217.

Veríssimo, A., J. R. McDowell & J. E. Graves, 2010. Global population structure of the spiny dogfish *Squalus acanthias*, a temperate shark with an antitropical distribution. Molecular Ecology 19 (8): 1651-1662.

Ward, R. D., T. S. Zemlak, B. H. Innes, P. R. Last & P. D. N. Hebert, 2005. DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 360: 1847-1857.

Ward, R. D., B. H. Holmes, W. T. White & P. R. Last, 2008. DNA barcoding Australasian chondrichthyans: Results and potential uses in conservation. Marine and Freshwater Research 59 (1): 57-71.

Ward, R. D., R. Hanner & P. D. N. Hebert, 2009. The campaign to DNA barcode all fishes, FISH-BOL. Journal of Fish Biology 74 (2): 329-356.

Yang, L., K. A. Matthes-Rosana & G. J. P. Naylor, 2014. Determination of complete mitochondrial genome sequence from the holotype of the southern Mandarin dogfish *Cirrhigaleus australis* (Elasmobranchii: Squalidae). Mitochondrial DNA 1736: 1-2.

Yang, L., K. A. Matthes-Rosana & G. J. P. Naylor, 2016. Complete mitochondrial genome of the blacknose shark *Carcharhinus acronotus* (Elasmobranchii: Carcharhinidae). Mitochondrial DNA 27 (1): 169-70.

Yano, K., J. D. Stevens & L. J. V. Compagno, 2004. A review of the systematics of the sleeper shark genus Somniosus with redescriptions of *Somniosus* (*Somniosus*) *antarcticus* and *Somniosus* (*Rhinoscymnus*) *longus* (Squaliformes: Somniosidae). Ichthyological Research 51 (4): 360-373.

Yano, K., J. D. Stevens & L. J. V. Compagno, 2007. Distribution, reproduction and feeding of the Greenland shark *Somniosus* (*Somniosus*) *microcephalus*, with notes on two other sleeper sharks, *Somniosus* (*Somniosus*) *pacificus* and *Somniosus* (*Somniosus*) *antarcticus*. Journal of Fish Biology 70 (2): 374-390.

Appendix

Appendix I Long-range polymerase chain reaction (PCR) protocol used in this study. The protocol shows the development of the primers followed by the settings used to perform the PCR reactions.

1. *Primer development*

For Greenland shark we designed the following primer sets:

Primer set	Primer name	Sequence	Base length	Tm °C	GC %
1A	Som_F1	5´-GATACCCTACTATGCCCAACCACAAACTTAGAC-3´	33	68.4	45.5
	Som_R1A	5´-GCATATCACTAAGGGTGGTAGGGAGTCA-3´	28	67.1	50.0
2A	Som_F2A	5´-AGATGCAGCCTCCCCAGTTATGGAAGAAC-3´	29	72.0	51.7
	Som_R2	5´-TAGAGTGGAGTAGGGCAGAGACTGGCGT-3´	28	70.8	57.1
3	Som_F3	5´-CTCTTGGTGCAACTCCAAGCAAGAGCTATGA-3´	31	72.9	48.4
	Som_R3	5´-GATGCAAAAGGTACGAGGTTGAGTCTCTGC-3´	30	71.0	50.0

We used the reference sequences of Pacific sleeper shark (*Somniosus pacificus*) and Spiny dogfish (*Squalus acanthias*) in order to find conserved regions for designing those primers. The reason for choosing those two species was their close relation to the Greenland shark (*Somniosus microcephalus*).

2. *Long-range PCR reaction step up using Phusion Hot Start II-Fidelity PCR Master Mix*

Gently vortex and briefly centrifuge all solutions after thawing. Set up the PCR reaction on ice. Setting the reaction up at room temperature may result in primer degradation by 3'→5' exonuclease activity of the enzyme mix.

To prepare several parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix by adding water, 2X Phusion (Thermo Scientific) and primers. Prepare enough master mix for the number of reactions and add one extra to compensate for pipetting errors. Aliquot the master mix into individual PCR tubes and add template DNA.

1. Gently vortex and briefly centrifuge all solutions after thawing.

2. Place a thin-walled PCR tube on ice and add the following components in the order listed in Table 1 for each 10 μl, 25 μl or 30 μl reaction:

	Volume to add to the reaction (μL)		
Components	10 μl reaction	25 μl reaction	30 μl reaction
H₂O	add to 2	add to 5	add to 6
2X Phusion HS II HF Master Mix	5	12.5	15
Forward primer	1	2.5	3
Reverse primer	1	2.5	3
DNA template	1	2.5	3
Total	10	25	30

3. Gently vortex and briefly centrifuge to collect all drops

4. Place the samples in a cycler and immediately start PCR.

PCR program (Three step-cycling protocol):

Cycle step	Temperature (ºC)	Time	Cycles
Initial denaturation	98	30 s	1
Denaturation	98	7 s	
Annealing	71	20 s	35
Extension/Elongation	72	2 min	
Final extension	72	7 min	1
Hold	4	∞	

The success of the PCR and the DNA fragment size was assessed using 0.8 % agarose gels and 2 kb size standard. The gels were run for 25 minutes at 160 V.

Appendix II Figure showing the workflow made from the DNA extraction to the next generation sequencing (NGS) in this study.
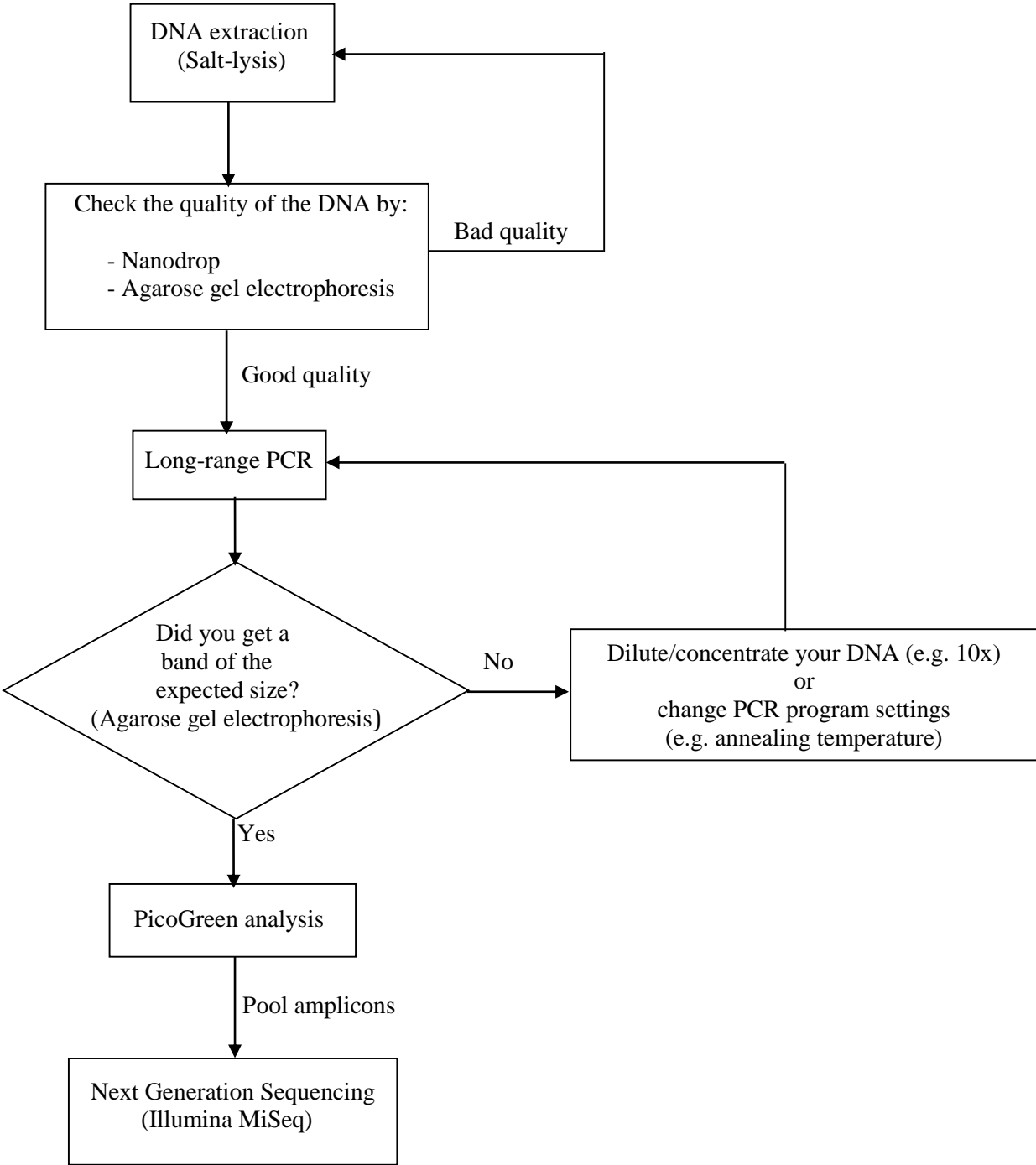
Appendix III Figure showing the workflow made from the DNA extraction to the Sanger sequencing in this study.
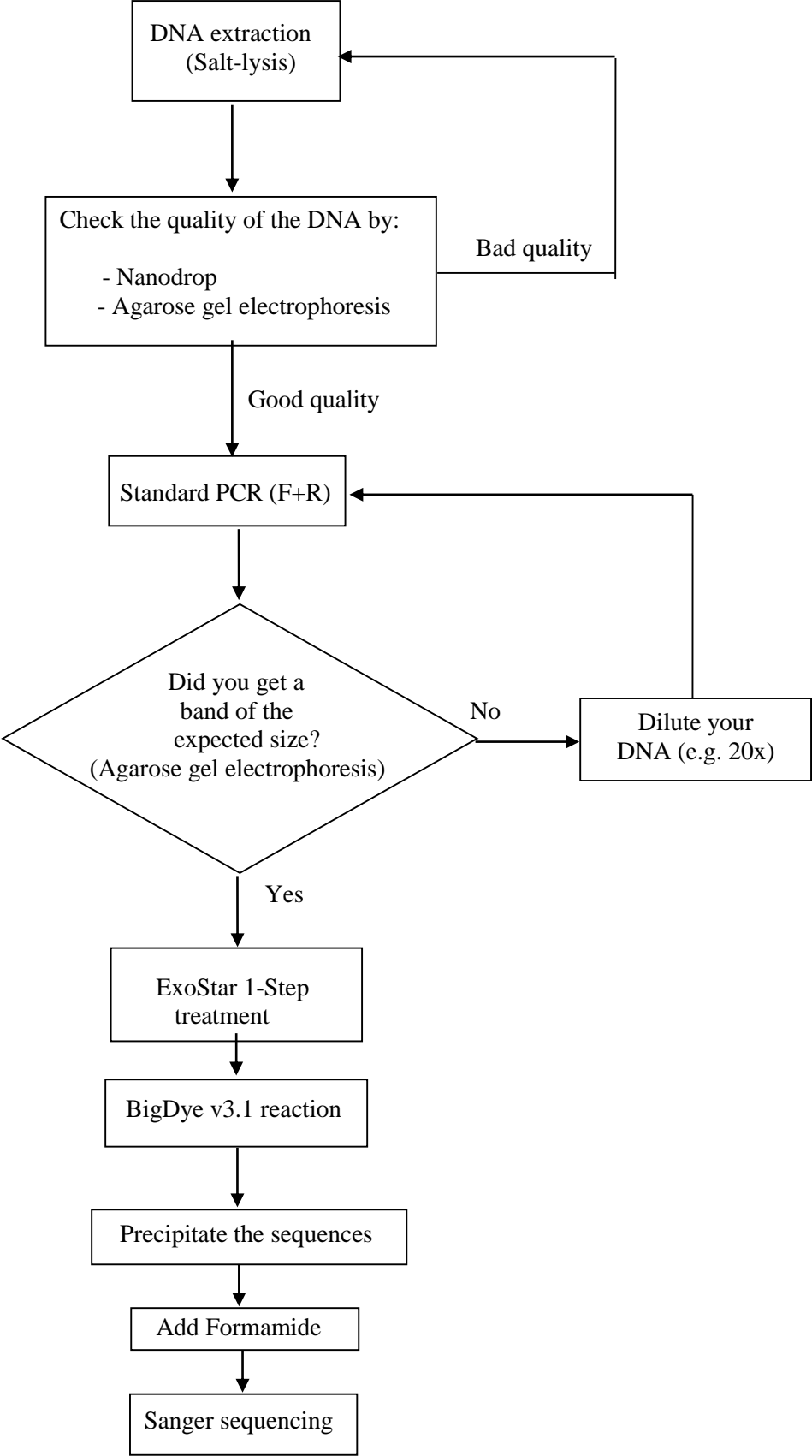
Appendix IV Table showing the fossil records used to infer the time-tree of analyzed species. The calibration point of most recent common ancestor (MRCA) by minimum and maximum age measured in million years ago (Mya).

Calibration point	Fossil - minimum age	Age (Mya)	References	Fossil-soft upper bound	Age (Mya)	References
MRCA of Batoids and sharks	fossil Synechodontiformes	204	Underwood (2006)	oldest chimaeroid	374	Cappetta (1993)
MRCA of Squalimorpha	Hexanchidae s.l	190	Underwood (2006)	fossil Synechodontiformes	204	Underwood (2006)
MRCA of Squatiniformes	*Squatina* sp.	151	Underwood (2006)	fossil Synechodontiformes	204	Underwood (2006)
MRCA of Squliformes	*Protosqualus*	130	Klug & Kriwet (2010)	*Squatina* sp.	151	Underwood (2006)
MRCA of Squalidae	*Squalus*	98	Klug & Kriwet (2010)	*Protosqualus*	130	Klug & Kriwet (2010)

Appendix V Table showing the comparison between all analyzed species mitogenomes depending on their nucleotide similarities. The color gradient indicates the nucleotide similarity from low (white) to high (black) percentage.

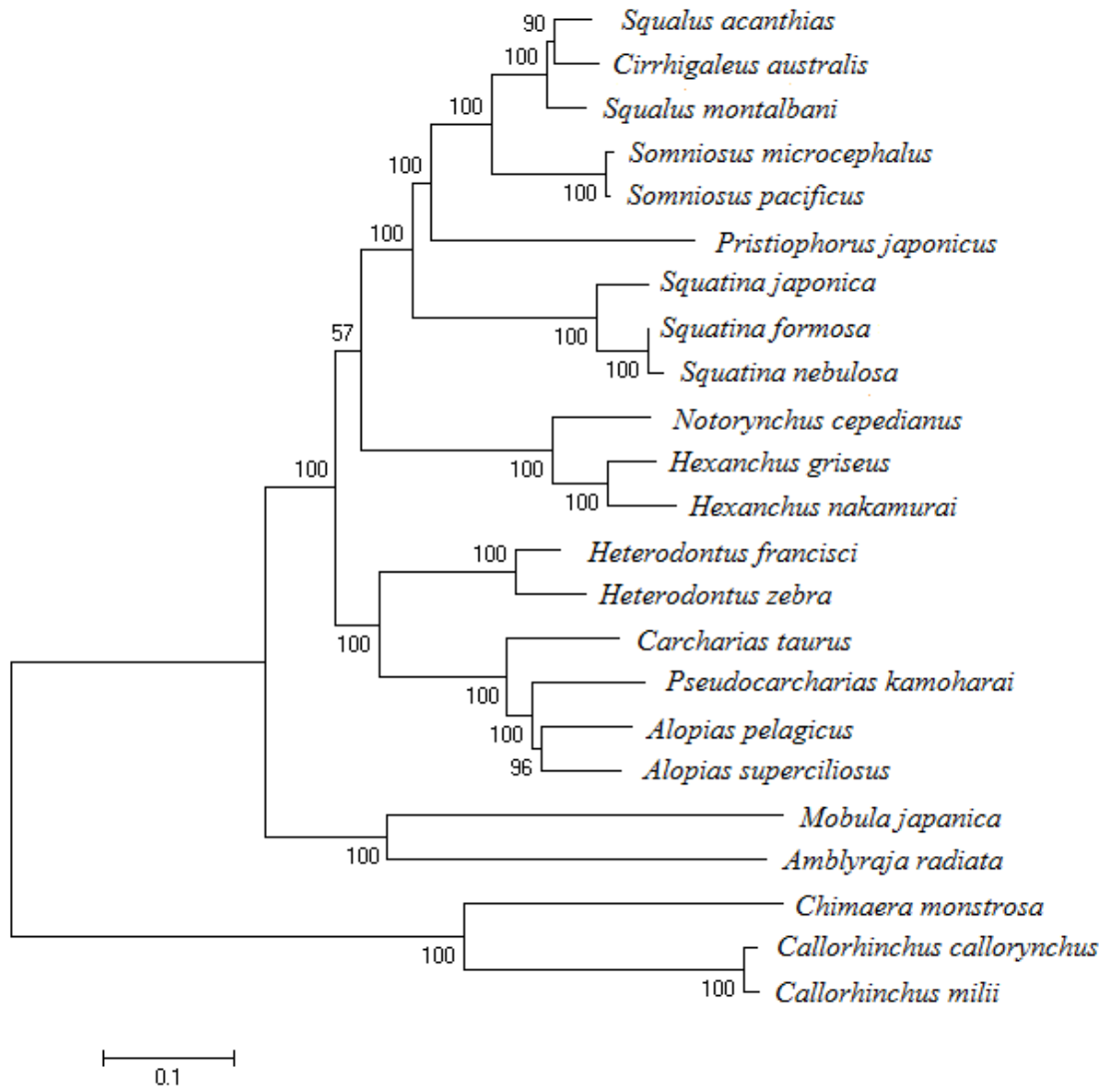|	S. microcephalus	S. pacificus	S. acanthias	S. montalbani	C. australis	S. formosa	S. japonica	S. nebulosa	P. japonicus	H. francisci	H. zebra	H. griseus	N. cepedianus	H. nakamurai	A. pelagicus	A. superciliosus	C. taurus	P. kamoharai	M. japonica	A. radiata	C. callorhinchus	C. milii	C. monstrosa
Somniosus microcephalus		99.0%	88.4%	87.5%	87.3%	82.2%	82.1%	81.8%	74.4%	80.9%	80.3%	77.8%	78.9%	71.7%	80.1%	79.9%	79.4%	79.7%	66.7%	75.2%	69.6%	69.6%	63.1%
Somniosus pacificus	99.0%		88.5%	87.6%	87.4%	82.2%	82.2%	81.8%	74.5%	81.1%	80.4%	77.8%	79.0%	71.8%	80.3%	80.1%	79.6%	79.9%	66.7%	75.3%	69.7%	69.7%	63.2%
Squalus acanthias	88.4%	88.5%		93.4%	93.4%	82.8%	82.7%	82.3%	74.9%	81.4%	80.8%	78.3%	79.1%	72.1%	80.7%	80.6%	79.7%	80.5%	66.9%	75.3%	70.0%	70.0%	63.5%
Squalus montalbani	87.5%	87.6%	93.4%		93.6%	82.2%	81.8%	81.8%	74.1%	80.9%	80.3%	78.0%	78.6%	71.7%	80.3%	80.1%	79.3%	80.1%	66.7%	75.0%	69.8%	69.8%	63.2%
Cirrhigaleus australis	87.3%	87.4%	93.4%	93.6%		82.2%	82.1%	81.7%	74.3%	80.9%	80.3%	77.8%	78.6%	71.7%	80.0%	79.7%	79.1%	79.9%	66.5%	75.1%	69.8%	69.7%	63.1%
Squatina formosa	82.2%	82.2%	82.8%	82.2%	82.2%		93.0%	92.0%	72.8%	80.0%	79.5%	77.2%	78.1%	71.1%	79.4%	79.2%	78.5%	79.5%	66.4%	74.8%	70.0%	70.0%	63.2%
Squatina japonica	82.1%	82.2%	82.7%	81.8%	82.1%	93.0%		98.6%	72.9%	80.0%	79.7%	77.0%	78.2%	71.1%	79.5%	79.3%	78.6%	79.7%	66.4%	74.7%	70.0%	70.0%	63.2%
Squatina nebulosa	81.8%	81.8%	82.3%	81.8%	81.7%	92.0%	98.6%		72.5%	79.5%	79.0%	76.7%	77.6%	70.7%	79.0%	78.8%	78.1%	79.0%	66.2%	74.4%	69.8%	69.8%	63.0%
Pristiophorus japonicus	74.4%	74.5%	74.9%	74.1%	74.3%	72.8%	72.9%	72.5%		72.4%	72.1%	71.0%	71.7%	71.2%	71.5%	70.9%	70.8%	70.8%	65.4%	67.3%	61.6%	61.5%	62.7%
Heterodontus francisci	80.9%	81.1%	81.4%	80.9%	80.9%	80.0%	80.0%	79.5%	72.4%		92.4%	77.4%	78.7%	71.2%	82.2%	81.8%	81.8%	81.8%	67.4%	75.8%	69.6%	69.6%	63.6%
Heterodontus zebra	80.3%	80.4%	80.8%	80.3%	80.3%	79.5%	79.7%	79.0%	72.1%	92.4%		76.7%	78.4%	71.3%	81.9%	81.7%	81.5%	81.3%	67.1%	75.7%	69.4%	69.4%	63.6%
Hexanchus griseus	77.8%	77.8%	78.3%	78.0%	77.8%	77.2%	77.0%	76.7%	71.0%	77.4%	76.7%		86.1%	85.1%	77.0%	76.7%	76.1%	77.3%	67.0%	72.6%	68.4%	68.4%	64.3%
Notorynchus cepedianus	78.9%	79.0%	79.1%	78.6%	78.6%	78.1%	78.2%	77.6%	71.7%	78.7%	78.4%	86.1%		79.0%	77.9%	77.8%	77.3%	78.1%	65.6%	73.5%	68.9%	68.7%	63.3%
Hexanchus nakamurai	71.7%	71.8%	72.1%	71.7%	71.7%	71.1%	71.1%	70.7%	71.2%	71.2%	71.3%	85.1%	79.0%		71.2%	70.7%	70.3%	71.1%	65.6%	67.0%	63.3%	63.2%	63.7%
Alopias pelagicus	80.1%	80.3%	80.7%	80.3%	80.0%	79.4%	79.5%	79.0%	71.5%	82.2%	81.9%	77.0%	77.9%	71.2%		89.6%	87.4%	88.8%	66.9%	75.1%	69.6%	69.5%	63.2%
Alopias superciliosus	79.9%	80.1%	80.6%	80.1%	79.7%	79.2%	79.3%	78.8%	70.9%	81.8%	81.7%	76.7%	77.8%	70.7%	89.6%		87.8%	89.1%	66.4%	75.2%	69.6%	69.5%	63.3%
Carcharias taurus	79.4%	79.6%	79.7%	79.3%	79.1%	78.5%	78.6%	78.1%	70.8%	81.8%	81.5%	76.1%	77.3%	70.3%	87.4%	87.8%		86.8%	66.2%	74.8%	69.2%	69.2%	63.1%
Pseudocarcharias kamoharai	79.7%	79.9%	80.5%	80.1%	79.9%	79.5%	79.7%	79.0%	70.8%	81.8%	81.3%	77.3%	78.1%	71.1%	88.8%	89.1%	86.8%		66.2%	74.8%	69.2%	69.2%	63.0%
Mobula japonica	66.7%	66.7%	66.9%	66.7%	66.5%	66.4%	66.4%	66.2%	65.4%	67.4%	67.1%	67.0%	65.6%	65.6%	66.9%	66.4%	66.2%	66.2%		66.6%	61.0%	61.0%	61.2%
Amblyraja radiata	75.2%	75.3%	75.3%	75.0%	75.1%	74.8%	74.7%	74.4%	67.3%	75.8%	75.7%	72.6%	73.5%	67.0%	75.1%	75.2%	74.8%	74.8%	66.6%		68.6%	68.5%	61.9%
Callorhinchus callorhinchus	69.6%	69.7%	70.0%	69.8%	69.8%	70.0%	70.0%	69.8%	61.6%	69.6%	69.4%	68.4%	68.9%	63.3%	69.6%	69.6%	69.2%	69.2%	61.0%	68.6%		98.0%	69.9%
Callorhinchus milii	69.6%	69.7%	70.0%	69.8%	69.7%	70.0%	70.0%	69.8%	61.5%	69.6%	69.4%	68.5%	68.7%	63.2%	69.5%	69.5%	69.2%	69.2%	61.0%	68.5%	98.0%		70.0%
Chimaera monstrosa	63.1%	63.2%	63.5%	63.2%	63.1%	63.2%	63.2%	63.0%	62.7%	63.6%	63.6%	64.3%	63.3%	63.7%	63.2%	63.3%	63.1%	63.0%	61.2%	61.9%	69.9%	70.0%	

Appendix VI Neighbor joining (A) and maximum likelihood (B) trees of analyzed sharks having Batoids and Chimaeras as outgroups. The numbers on the branches indicate posterior probabilities in percentage. Branch lengths are proportional to the amount of genetic changes (nucleotide substitutions per site).

A

B

Squalus acanthias — 90
Cirrhigaleus australis — 100
Squalus montalbani — 100
Somniosus microcephalus — 100
Somniosus pacificus
Pristiophorus japonicus
Squatina japonica
Squatina formosa — 100
Squatina nebulosa — 100
Notorynchus cepedianus
Hexanchus griseus — 100
Hexanchus nakamurai — 100
Heterodontus francisci — 100
Heterodontus zebra
Carcharias taurus
Pseudocarcharias kamoharai — 100
Alopias pelagicus — 100
Alopias superciliosus — 96
Mobula japanica
Amblyraja radiata — 100
Chimaera monstrosa
Callorhinchus callorynchus — 100
Callorhinchus milii — 100

100
57
100
100
100

0.1

Appendix VII Time-tree inferred by ML analyses using Bering strait geological event calibrations. It shows estimated divergence times of analyzed species. The outgroup appears as a grey line comprising the three species of Chimaeras (*Callorhinchus callorynchus*, *Callorhinchus milii*, *Chimaera monstrosa*). Branch lengths are proportional to divergence times measured by million years ago (Mya). Above the time scale, divisions of geologic periods are indicated in different colors.