

Article



# Complete Mitochondrial DNA Genome of Nine Species of Sharks and Rays and Their Phylogenetic Placement among Modern Elasmobranchs

Vasiliki Kousteni<sup>1</sup>, Sofia Mazzoleni<sup>1</sup>, Katerina Vasileiadou<sup>1,2</sup> and Michail Rovatsos<sup>1,\*</sup>

- <sup>1</sup> Department of Ecology, Faculty of Science, Charles University, Viničná 7, 12800 Prague, Czech Republic; kousteni@inale.gr (V.K.), sofia.mazzoleni@natur.cuni.cz (S.M.), kvasileiadou@hcmr.gr (K.V.)
- <sup>2</sup> Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Thalassocosmos, P.O. Box 2214, 71003 Heraklion, Crete, Greece
- \* Correspondence: michail.rovatsos@natur.cuni.cz; Tel.: +42-02-2195-1758

Abstract: Chondrichthyes occupy a key position in the phylogeny of vertebrates. The complete sequence of the mitochondrial genome (mitogenome) of four species of sharks and five species of rays was obtained by whole genome sequencing (DNA-seq) in the Illumina HiSeq2500 platform. The arrangement and features of the genes in the assembled mitogenomes were identical to those found in vertebrates. Both Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were used to reconstruct the phylogenetic relationships among 172 species (including 163 mitogenomes retrieved from GenBank) based on the concatenated dataset of 13 individual protein coding genes. Both ML and BI analyses did not support the "Hypnosqualea" hypothesis and confirmed the monophyly of sharks and rays. The broad notion in shark phylogeny, namely the division of sharks into Galeomorphii and Squalomorphii and the monophyly of the eight shark orders, was also supported. The phylogenetic placement of all nine species sequenced in this study produced high statistical support values. The present study expands our knowledge on the systematics, genetic differentiation, and conservation genetics of the species studied, and contributes to our understanding of the evolutionary history of Chondrichthyes.

Keywords: Chondrichthyes; complete mitogenome; phylogeny; rays; sharks; systematics

## 1. Introduction

Cartilaginous fish (Class Chondrichthyes) consist a group of vertebrates that demonstrate an old radiation, dating back about 400 million years [1,2]. This lineage has survived four mass extinction events [1] and most present-day taxa derive from Mesozoic forms [3]. The evolutionary success of Chondrichthyes is partly due to the efficiency of their *K*selective reproductive traits [4], such as large body size, slow growth rate, late maturity, low fecundity and large offspring size [5–7]. The class Chondrichthyes, which comprises the most diverse group of large predatory animals, currently includes 14 orders, 60 families and 198 genera with approximately 1200 species. It is composed of two subclasses, the Holocephali (chimaeras) including one superorder (Holocephalimorpha with 49 species), and Elasmobranchii including three superorders: Galeomorphii and Squalomorphii with 347 and 157 shark species, respectively, and Batoidea with 639 species of rays, stingrays, skates and sawfishes [8–10].

Chondrichthyes play an important ecological role, most notably functioning as either top predators with top-down control on the size and dynamics of many species [11] or mesopredators in the marine food webs [12,13] by linking different trophic levels in the marine ecosystems and contributing to system dynamics and stability [14]. Furthermore, apart from providing an important perspective to interpreting functional and life-history

Citation: Kousteni, V.; Mazzoleni, S.; Vasileiadou, K.; Rovatsos, M. Complete Mitochondrial DNA Genome of Nine Species of Sharks and Rays and Their Phylogenetic Placement among Modern Elasmobranchs. *Genes* **2021**, *12*, 324. https://doi.org/10.3390/ genes12030324

Academic Editor: Antonio Carapelli Received: 30 January 2021 Accepted: 22 February 2021 Published: 24 February 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2021 by the author. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/). evolution as being the sister group to all other extant jawed vertebrates (Gnathostomata) [15], they exhibit a genomic architecture that is likely closer to the ancestral vertebrate condition compared to teleosts [16]. Their commercial value, especially of their meat, fin and liver is increasing as targeted teleost fish become less accessible [17,18]. As a result, overfishing has profoundly altered shark and ray populations [19–21] and several species are facing a two-fold higher extinction risk compared to finfish [22]. The International Union for the Conservation of Nature (IUCN) has assessed the conservation status of 604 out of 1192 taxonomically valid species with 179 chondrichthyans categorized as threat-ened (Vulnerable—VU; Endangered—EN; Critically Endangered—CR) [2]. Due to the fact that almost 1/3 of the assessed species face extinction risk [2,23] and further 63 Data Deficient (DD) species were predicted to be threatened based on correlates of IUCN threat status, chondrichthyans comprise the most-imperiled class among all vertebrates [23]. This highlights the importance of preserving their biodiversity and shedding light on their phylogenetic relationships [23] by prioritizing especially threatened species as they embody significant amounts of unique evolutionary history [2].

In recent years, with the advent of molecular data, there has been a significant effort towards elucidating the evolutionary history of chondrichthyans [2,15]. However, their phylogeny is still controversial at all levels, ranging from superorders to genera [24]. A significant obstacle towards resolving their phylogenetic relationships is that several species are likely to represent complexes of several distinct species that require taxonomic resolution, for example some dogfishes, eagle rays, and stingrays [24–26]. Moreover, although the "Hypnosqualea" hypothesis, suggesting that batoids are derived sharks related intimately to the sawfish and angel sharks [27–30], is no longer supported [31–33] and it is widely believed that modern sharks (Neoselachii) are monophyletic, the relationships among the three main superorders (Galeomorphii, Squalomorphii and Batoidea) and the arrangement of the orders within these groups remain unsolved [15]. Notably, most of the phylogenetic studies of chondrichthyans are based on few nuclear and/or mitochondrial DNA (mtDNA) genes [2,31,34]. Individual mitochondrial markers are widely used to assess species diversity and population connectivity [35–37] due to the relatively simple sequencing procedures and the high rates of nucleotide substitution [38].

The recent blooming of Next Generation Sequencing (NGS) methodologies allowed the reliable and accurate assembly of the complete mtDNA genomes (mitogenomes) for phylogenetic analysis [15,23,39]. Currently, the complete mitogenomes have been sequenced, assembled and annotated from 82 species of sharks, 73 species of rays and 8 species of chimaeras (Table S1).

In this context, the principal objective of the present study was to sequence and characterize the complete mitogenome of nine chondrichthyans (four sharks and five rays) sampled as bycatch by commercial fisheries in Greece. The gene content, organization, codon usage and base composition were analyzed in each assembled mitogenome. The phylogenetic relations of all 172 species were reconstructed based on Maximum Likelihood and Bayesian Inference methods that were applied to the concatenated sequences of the 13 protein coding genes of the mitogenome.

## 2. Materials and Methods

## 2.1. Tissue Sampling and DNA Extraction

Individual fin clips were obtained from four shark species (*Galeus melastomus, Odontaspis ferox, Prionace glauca* and *Squalus blainville*) and five ray species (*Bathytoshia centroura, Dasyatis tortonesei, Gymnura altavela, Raja undulata* and *Torpedo marmorata*) (Table 1). All specimens were incidentally caught by commercial trawlers or long-liners in the Aegean Sea, Greece and were kindly provided by fishermen. The experimental design was performed by an accredited researcher (MR: CZ03540) and was approved by the ethical committee of the Faculty of Science, Charles University, Czech Republic (UKPRF/28830/2021). Fin samples were preserved in 95% ethanol and stored at -20°C. Total genomic DNA was extracted from approximately 25 mg of each sample using the standard protocol of the DNeasy Tissue kit (Qiagen, Chatsworth, CA, USA). The DNA concentration of each sample was estimated using NanoDrop One Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA fragmentation was checked with 1% agarose gel electrophoresis. Species identification was initially assessed macroscopically based on standard taxonomic features [9] and was confirmed by blasting the obtained cytochrome *c* oxidase subunit I (*COI*) gene from each assembled mitogenome to the homologous sequences deposited in GenBank, using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) [40].

**Table 1.** The complete mitogenome was assembled and annotated from four shark species (*Galeus melastomus*, *Odontaspis ferox*, *Prionace glauca* and *Squalus blainville*) and five ray species (*Bathytoshia centroura*, *Dasyatis tortonesei*, *Gymnura altavela*, *Raja undulata* and *Torpedo marmorata*) All information for the mitogenome sequence and annotation is deposited in the GenBank database under the accession numbers MT274568–MT274575. The IUCN conservation status globally and for the Mediterranean Sea is indicated per species. Abbreviations: CR, Critically Endangered; DD, Data Deficient; EN, Endangered; LC, Least Concern; NT, Near Threatened; VU, Vulnerable.

Sussia	Common Nome	Jame IUCN Conservation Status	Conservation Status	GenBank
Species	Common Name	Globally	Mediterranean Sea	Accession Number
Bathytoshia centroura	Roughtail stingray	VU	Not evaluated	MT274568
Dasyatis tortonesei	Tortonese's stingray	DD	VU	MT274569
Galeus melastomus	Blackmouth catshark	LC	LC	MT274570
Gymnura altavela	Spiny butterfly ray	VU	CR	MT274571
Odontaspis ferox	Smalltooth sand tiger	VU	CR	MT274572
Prionace glauca	Blue shark	NT	CR	MT274573
Raja undulata	Undulate ray	EN	NT	MT274574
Squalus blainville	Longnose spurdog	DD	DD	MT274575
Torpedo marmorata	Marbled electric ray	DD	LC	MT274576

#### 2.2. Sequencing and Mitogenome Assembly

Total DNA sequencing was performed by Novogene Bioinformatics Technology Co., Ltd. (Yuen Long, Hong Kong) in Illumina HiSeq2500 platform with 150 base pair per-end sequencing option. The reads were trimmed for adapters in Trimmomatic [41], checked for quality in FastQC [42] and mapped against a reference mitogenome from a closely related species in Geneious Prime software [43]. The reference mitogenomes are presented in Table S1. Subsequently, the mapped Illumina reads were de novo assembled with Geneious Prime [43] to reconstruct the complete mitogenome of each species. As a final step, the total DNA-seq reads were mapped to the assembled mitogenome from the same species with Geneious Prime [43] to confirm the quality of the assembly, correct potential assembly gaps, and finally close the circular molecule. The infrastructure of MetaCentrum (www.metacentrum.cz (accessed on 26/10/2020)) was used for computational resources.

#### 2.3. Annotation and Sequence Analysis

The complete mitogenome of each species was annotated using MitoAnnotator on the MitoFish website (http://mitofish.aori.u-tokyo.ac.jp/annotation/input.html (accessed on 26/10/2020)) [44]. The programs RNAmmer (http://www.cbs.dtu.dk/services/RNAmmer/ (accessed on 26/10/2020)) [45] and tRNA scan-SE (http://lowelab.ucsc.edu/tRNAscan-SE/ (accessed on 26/10/2020)) [46] were used to confirm the ribosomal RNA (rRNA) and the transfer RNAs (tRNAs) annotation results, respectively. The secondary structures of tRNAs were predicted by MITOS (http://mitos.bioinf.uni-leipzig.de/help.py (accessed on 26/10/2020)) [47]. The control region was inspected by the program "Tandem Repeats Finder" (https://tandem.bu.edu/trf/trf.html (accessed on 26/10/2020)) [48]. The boundaries of the protein coding genes (PCGs), rRNA genes and tRNA genes were refined manually by comparison with the annotated elasmobranch mitogenomes from GenBank. The obtained complete mitogenomes were deposited in GenBank under the accession numbers MT274568–MT274575 (Table 1; Table S1).

The nucleotide composition and the A+T and G+C contents (%) were calculated for the complete mitogenome and each of the 13 PCGs per species in MEGA v5.1 [49]. The AT skews and GC skews were calculated using the following formulas: AT skew D (A-T)/(A+T), GC skew D (G-C)/(G+C) [50]. Finally, the codon usage in the mitochondrial PCGs was estimated per species by the Sequence Manipulation Suite (http://www.bioinformatics.uni-muenster.de/tools/sms2/codon\_usage.html) [51]. The relative synonymous codon usage value (RSCU) of a codon, corresponding to the number of times that a codon appears in a gene in relation to the number of expected occurrences under an assumption of equal usage of synonymous codons (values less than 1 or more than 1 indicate that the codons are used less or more often than the expected) [52] was calculated in DNASP v6.12.03 [53].

## 2.4. Phylogenetic Analysis

For phylogenetic analyses, in addition to the nine assembled mitogenomes, a total of 163 complete mitochondrial genomes of sharks, rays and chimaeras were retrieved from GenBank (Table S1). The 13 PCGs were extracted, aligned with the CLUSTAL W algorithm [54], and concatenated using the software Geneious [43]. The most likely model of sequence evolution for each dataset (individual PCGs and concatenated dataset) was selected by JModelTest v2.1.7 [55], based on the Bayesian Information Criterion (Table S2). The phylogenetic relationships were initially reconstructed with the Maximum Likelihood (ML)-based approach through the online implementation of PhyML v3 provided by the Montpellier Bioinformatics Platform (http://www.atgc-montpellier.fr (accessed on 26/10/2020)) [56] with 1000 bootstrap replicates. In addition, a Bayesian Inference (BI) phylogenetic tree was constructed in MrBayes 3.2.6 [57] on the computer cluster MetaCentrum (www.metacentrum.cz (accessed on 26/10/2020)). The Bayesian Inference method was applied using the Markov Chain Monte Carlo (MCMC) algorithm from randomly generated starting trees for 5 million generations with trees sampled every 100 generations and 2 runs with 4 chains (2 heated and 2 cold). The first 25% of the trees were discarded as burn-in, and the remaining sampled trees were used to estimate the 50% majority rule consensus tree and the Bayesian posterior probabilities. For the concatenated analysis, the matrix was partitioned by gene to include gene-specific models of substitution. ML and Bayesian analyses were carried out for each individual PCG and the concatenated dataset of all 13 PCGs under the evolutionary models presented in Table S2. The Maximum Likelihood (ML) and Bayesian Inference (BI) trees were visualized and edited in FigTree v1.4.3 [58].

## 3. Results and Discussion

## 3.1. Genome Organization

The size of the assembled mitogenomes ranged from 16,682 bp in *Odontaspis ferox* to 19,472 bp in *Gymnura altavela* and was within the expected size range of the complete mtDNA sequences retrieved from GenBank (Table S1). To data the smallest mitogenome has been reported for the Chilean devil ray *Mobula tarapacana* (Philippi, 1892) [59] (15,686 bp; Accession number: MH669414) and the largest for the Pacific spookfish *Rhinochimaera pacifica* (Mitsukuri, 1895) (24,889 bp; Accession number: HM147141) [60]. The differences in the mtDNA genome size among elasmobranchs correspond mainly to the high content of tandem repeats in the control region [24].

The gene order and content of all the nine assembled mitogenomes was the typical expected for vertebrates [61]. Specifically, the mitogenome of each species contained 13 protein coding genes (PCGs), 2 ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA)

genes, the control region (*D-loop*) and several small noncoding regions. The analytical description of each of the nine assembled mitogenomes is presented in Table S3. Additionally, the gene map and a short description of each assembled mitogenome is presented in Figures S1-S9.

#### 3.2. Protein-Coding Genes and Codon Usage

The mitogenome of each species encoded a typical full set of 13 proteins. The majority of the PCGs were transcribed from the heavy (H) strand, except for the *ND6* gene and eight out of the 22 tRNA genes (tRNAGIn, tRNAAla, tRNAAsn, tRNACys, tRNATyr, RNASer, tRNAGlu and tRNAPro), which were transcribed from the light (L) strand. The start codons were the typical ATG codon of all PCGs, regardless of the species with some exceptions: the *COI* gene was initiated by a GTG codon, *ATP6* gene was initiated by a GTG codon only in *Torpedo marmorata*, and the *ND6* gene was initiated by a CTA or TTA codon. Among the mitochondrial PCGs, the ND5 was the longest, while the ATP8 was the shortest, in all species. Most of the PCGs were terminated by a complete (TAA/TAG), while the incomplete termination codon (TA/T) was found in 5 out of the 13 PCGs. Such incomplete termination codons (TA/T) are a common phenomenon in metazoan mitogenomes and can be extended to a complete TAA termination codon through polyadenylation of the 3'-end of the mRNA, which occurs after transcription [62]. These features of the initial and stop codons are commonly observed in elasmobranchs [39,63] and are similar to the majority of the vertebrate mitochondrial PCGs [64].

The base composition and the RSCU values of the mitochondrial PCGs are presented per species in Tables S4 and S5. Each PCG and the complete mitogenome of all species were rich in the A+T content, resulting in a strong bias towards A+T rich codons in the codon usage, which appears to be a shared feature in vertebrates [61]. The most frequently used codons across species were: TTALeu (average = 4.21%), TTTPhe (average = 3.17%), CTALeu (average = 3.14%), TATTyr (average = 2.90%), CTTLeu (average = 2.79%), CCTPro (average = 2.65%), ACAThr (average = 2.59%) and ATAMet (average = 2.54%). The codons with the highest RSCU values that were found in the PCGs from the nine assembled mitogenomes were TTALeu (RSCU average = 1.76), GCCAla (average = 1.56), TCTSer (average = 1.55), TCASer (average = 1.52) and AAALys (average = 1.52) (Table S5).

#### 3.3. rRNA and tRNA Genes

The mitogenome of each species contained 22 tRNA genes interspersed along the genome, the small subunit of rRNA (12S rRNA) and the large subunit of rRNA (16S rRNA). They were transcribed in the same direction on the H-strand and separated by tRNAVal. The size in base pairs (bp) of all tRNA and rRNA genes is presented in Table S2. All tRNA genes could fold into a distinctive cloverleaf secondary structure except tRNASer(AGY), which contained a simple loop without making the dihydrouridine arm, similarly to many metazoan mitogenomes [64,65] (Figure S10).

#### 3.4. Noncoding Regions

The noncoding regions included the origin of light strand replication (OL), one putative control region (*D-loop*) and intergenic spacers, namely some overlapping nucleotides and gaps between PCGs or between PCGs and tRNAs (Table S2). In each assembled mitogenomes the OL region was located between the tRNAAs and tRNACys genes and the control region was located between the tRNAPro and tRNAPhe genes. The OL region ranged in size from 32 bp in *Raja undulata* to 41 bp in *Galeus melastomus*. Moreover, the control region exhibited significant size variation among the studied species, ranging in size from 1068 bp in *Prionace glauca* to 3768 bp in *Gymnura altavela*, and was enriched in tandem repeat sequences in all species, except *Squalus blainville*. This finding confirms the fact that the control region exhibits extensive nucleotide and size polymorphism, as it has been shown in several elasmobranchs [66–68] and teleosts [69–71]. An analytical description of the tandem repeats is presented in Table S6.

#### 3.5. Phylogenetic Inference

Both ML and BI phylogenetic analyses supported the division of the class Chondrichthyes into four superorders (Galeomorphii, Squalomorphii, Batoidea and Holocephalimorpha) (Figures 1a,1b,2a,2b), but not the "Hypnosqualea" hypothesis, which, based on morphological traits, suggests that Batoidea is part of the shark group [27–30]. The monophyly of modern sharks has been proposed previously by Maisey [72] who morphologically identified three groups of Chondrichthyes, the first showing an orbitostylic jaw suspension (Hexanchiformes, Squaliformes, Pristiophoriformes and Squatiniformes), the second group represented by the galeomorphs (Heterodontiformes, Orectolobiformes, Lamniformes and Carcharhiniformes) and the third group with all batoids. Such classification was further supported by morphological [73,74] and recently, by molecular studies [2,8,15,31–34,75–77], reinforcing the scenario that the "Hypnosqualea" morphological traits could be regarded as homoplasy, due to the convergent adaptation to the benthic life [31,75].

The phylogenetic placement of the studied species was supported with high bootstrap and posterior probability values based on both ML and BI phylogenetic analyses (Figures 1a,b, 2a,b), and notably corresponds to their reproductive mode. Galeus melastomus was placed within Pentanchidae (Carcharhiniformes) with oviparity as mode of reproduction [4]. Prionace glauca was placed within Carcharhinidae (Carcharhiniformes) with placental viviparity as mode of reproduction [4]. Odontaspis ferox was placed within the order Lanmniformes with oophagy (type of aplacental viviparity supported by yolk and maternal contribution) as the sole mode of reproduction [78]. Squalus blainville was placed within Squalidae (Squaliformes) with yolk sac viviparity (a type of aplacental viviparity where embryos feed solely on yolk) as the mode of reproduction of all Squalomorphii [4]. Bathytoshia centroura (Dasyatidae), Dasyatis tortonesei (Dasyatidae) and Gymnura altavela (Gymnuridae) were placed within Myliobatiformes with istotrophy (a type of aplacental viviparity supported by yolk and uterine milk) as the sole mode of reproduction [79]. Finally, Torpedo marmorata was placed within Torpedinidae (Torpediniformes) reproducing with istotrophy [80] and as a sister taxon to Narcinidae (Torpediniformes) reproducing with yolk sac viviparity with other maternal contribution [81]. The effect of the reproductive mode on the phylogenetic placement of elasmobranchs has also been supported by Hull et al. [82] who showed that Mustelus mustelus was phylogenetically closer to the placental species Mustelus griseus, both of which are viviparous placental, in contrast to Mustelus manazo, which is aplacental [83,84]. Furthermore, given that oviparity is the sole reproductive mode for all Chimaeriformes and Heterondontiformes species, and for some families of Orectolobiformes (Parascylliidae, Hemiscylliidae and Stegostomatidae), Carcharhiniformes (Scyliorhinidae and Proscyllidae) and Rajiformes (Rajidae) [4], earlier suggestions that egg-laying oviparous sharks are ancestral [81,85,86] are confirmed.



а



Carcharhinus leucas (KF646785) Carcharhinus amboinensis (KM21745) Carcharhinus arcnotus (KN248470) Carcharhinus longinanus (KN248470) Carcharhinus longinanus (KN24470) Carcharhinus longinanus (KN24470) Carcharhinus solscurus (KA247054) Carcharhinus solscurus (KA247054) Carcharhinus solscurus (KA247054) Carcharhinus ausoti (K1865755) Carcharhinus dussumieri (K18091436) Carcharhinus dussumieri (K18091436) Carcharhinus sorrah (KA65755) Carcharhinus sorrah (KA65755) Carcharhinus sorrah (KA65755) Carcharhinus ambloty (K18092088) Carcharhinus ambloty (K198052) Giphis invessi (K17698052) Solidon ancorhynchos (G0693102) Solidon ancorhynchos (G0693102) Solidon ancorhynchos (G0693102) Biphyra ibenatus (K1347599) Galaecerdo cuvier (K11728) Hemigaleus microstoma (K1003687) Hemirakis (Ix036547) Loxodon macrothus (K1347599) Galaecerdo cuvier (K170393) Halaelurus buergeri (KU892589) Galeus melasotus (M1831446) Seyliorhinus canzula (Y10667) Hustelus maszo (AB015962) Halenitrakis (K734759) Proseyllium habereri (KU721838) Seyliorhinus canzau (K1004573) Halaelurus buergeri (KU892589) Galeus melasomus (K734759) Proseyllium ambratilic (K100366) Proderma patherinum (M1831446) Seyliorhinus canzula (Y16067) Lurus ayautis (K561042) Carcharios (K561042) Carcharios (K561043) Laman ansi (K5610464) Cetorhinus maximus (K759703) Carcharis kamoharai (K057315) Halaelurus buergeri (KU72133) Alopias vupinus (K759703) Carcharis kamoharai (K195351) Hustelus maximus (K759703) Carcharis kamoharai (K195357) Alopias vupinus (K759703) Carcharis kamoharai (K1955726) Alopias vupinus (K759703) Carcharis kamoharai (K1955726) Alopias vupinus (K759703) Carcharis kamoharai (K1955726) Alopias vupinus (K759703) Carcharis kamoharai (K195572) Chilosyllium paloisum (K759733) Carcharis kamoharai (K195575) Alopias vupinus (K7597572) Cephalosyllium contat

3) Ca	rcharhiniformes	ıobranchii
6) La	mniformes	Elasn
Or	ectolobiformes	
l He	terodontiformes	
Sq	ualiformes	
Sq	uatiniformes	
1) I He	istiophoriformes xanchiformes	



**Figure 1. (a)** Bayesian mitogenomic phylogeny of Selachii based on the concatenated dataset of 13 protein-coding genes. (b) Bayesian mitogenomic phylogeny of Batoidea and Holocephali, based on the concatenated dataset of 13 protein-coding genes. Posterior probabilities are presented next to the nodes. The complete phylogeny is illustrated in the miniphoto. Mitogenomes assembled for the first time in this study are indicated by an asterisk (\*).







Carcharhiniformes

Elasmobranchii

Lamniformes

Squaliformes

Squatiniformes

- Pristiophoriformes Hexanchiformes
- | Heterodontiformes

Orectolobiformes

а



**Figure 2. (a)** Maximum Likelihood mitogenomic phylogeny of Selachii based on the concatenated dataset of 13 proteincoding genes. (b) Maximum Likelihood mitogenomic phylogeny of Batoidea and Holocephali, based on the concatenated dataset of 13 protein-coding genes. Bootstrap values are presented next to the nodes. The complete phylogeny is illustrated in the miniphoto. Mitogenomes assembled for the first time in this study are indicated by an asterisk (\*).

> Within Selachii, the phylogenetic analysis recovered the eight well-known shark orders divided into two distinct clades, the Squalomorphii and the Galeomorphii, being consistent with previous morphological [30–37] and molecular divisions [2,8,15,24,31– 33,75,77]. The Squalomorphii was represented by four orders. According to the Bayesian mitogenomic phylogeny (Figure 1a), the monophyletic Hexanchiformes was placed at the most basal position and sequentially followed by Squatiniformes placed as sister taxon to Pristiophoriformes, and the monophyletic Squaliformes that was placed as a sister taxon to the Squatiniformes-Pristiophoriformes group. The proposed topology is supported

with high posterior probability values and is consistent with previous molecular studies [15,24,31–34,87]. Molecular [77] and morphological studies [88] have placed Squaliformes as a sister taxon to Pristiophoriformes, while Heinicke et al. [8] suggested a sister relationship between Squaliformes and Squatiniformes. Pristiophoriformes are strongly supported as squaloidlike sharks based on morphological studies [27–29] by lacking the eight batoid synapomorphies [89]. Our findings also support the placement of Squatiniformes within squalimorphs, in contrast to Compagno [90] who proposed four superorders (Galeomorphii, Squalomorphii, Squatinimorphii and Batoidea). The placement of Squatiniformes and Pristiophoriformes within squalimorphs has been supported since the first extensive molecular phylogeny of Douady et al. [31], based on a fragment of the mitochondrial 12S, 16S and transfer RNA valine genes (2400 nucleotides) from over 20 elasmobranchs, until more recent studies that analyzed the complete mitogenome of more than 70 elasmobranchs [15,77].

Within Hexanchiformes, Hexanchinidae was placed as a sister taxon to Chlamydoselachidae. Within Squatiniformes, the basal *Squatina squatina* was grouped with *Squatina japonica* and sequentially by *Squatina nebulosa* which was placed as sister species to *Squatina formosa*. Squatinidae was placed as a sister taxon to Pristiophoridae represented by *Pristiophorus japonicus*. Our findings support the paraphyletism of Squatiniformes and Pristiophoriformes similarly to previous studies based on both molecular [8,15,24,33] and morphological traits [72], and define the position of *Squatina nebulosa* in contrast to Maisey [72].

Within Squaliformes, Etmopteridae was placed as a sister taxon to Dalatiidae, and both families were at the most basal position of the order. Somniosidae was placed as a sister taxon to Squalidae. Squalidae showed monophyly as in Vélez-Zuazo and Agnarsson [33] where Squalimorphii were represented by more species and families. According to Vélez-Zuazo and Agnarsson [33] Somniosidae, Dalatiidae and Etmopteridae showed paraphyly, a notion that was partially confirmed in the present study, probably due to the different representation of the families. Vélez-Zuazo and Agnarsson [33] observed poor support for most of the relationships among the families of Squaliformes, while in the present study they were strongly supported. Notably, a distinct phylogenetic placement of *Squalus* species was supported by high posterior probability values, with *Squalus acanthias* recovered as the basal taxon within Squalidae. Squaliformes comprise the second most diverse order of sharks with taxonomic uncertainties within the genus *Squalus* [37]. Thus, by providing the complete mitogenome of *Squalus Blainville*, the present study could support future phylogenetic studies with the scope to resolve these uncertainties.

The Galeomorphii was represented by four monophyletic orders (Figure 1a). The basal Heterodontiformes followed by Orectolobiformes and, sequentially, by the Carcharhiniformes-Lamniformes group, a topology previously supported by morphological [28,29] and molecular studies [2,8,15,24,77]. Nevertheless, based on ML tree topology, Orectolobiformes was placed at the most basal position of a group including all of the sharks' orders (Figure 2a). Based on BI analysis, the phylogenetic placement of Heterodontiformes as sister taxon to all other galeomorphs has already been reported [8,15,29,77,88] and opposes the close relationship between Heterodontiformes and squalimorphs suggested by Mallatt and Winchell [87] and Human et al. [32]. Moreover, the basal placement of Heterodontiformes represented by the monophyletic genus Heterodontus, opposes previous studies suggesting the galeomorph Heterodontus and the squalimorph Chlamydoselachus as basal neoselachians or nonneoselachian sharks [91,92]. Additionally, the Lamniformes-Carcharhinifromes grouping opposes the Lamniformes-Orectolobiformes grouping that was suggested based on sequences of the mtDNA and/or nuclear genes [31–33,76,77,87]. Winchell et al. [75] used nuclear major and minor rRNA subunits to relate the Lamniformes–Orectolobiformes group to the Isurida group that was initially proposed based on morphological similarities [93], a view not corroborated by Compagno [88]. Most of these similarities were considered by Winchell et al. [75] as homoplasy and were treated as convergent adaptations for pelagic carnivory, a view that cannot be confirmed in recent molecular phylogenetic studies [2,15,24,77].

Within Orectolobiformes, Orectolobidae was placed in a basal position related to the group formed by all other families similarly to previous studies [15,24,77]. Stegostomatidae was placed as a sister taxon to the Rhincodontidae–Ginglymostomatidae group and Hemiscylliidae was placed as a sister taxon to the Stegostomatidae–Rhincodontidae– Ginglymostomatidae group. An identical placement of the families within Orectolobiformes has been found in previous phylogenetic studies [15,33,77].

Lamniformes was represented by seven families, including the paraphyletic Alopiidae-Megachasmidae and Odontaspididae (Figure 1a). Mitsukurinidae was placed at the most basal position of all lamniforms divided into two groups: the first group formed by the basal Odontaspididae (Carcharias taurus), Cetorhinidae and Lamnidae and the second group formed by the basal Alopias superciliosus placed as a sister taxon to a group subdivided to the other representatives of the Alopiidae family and the Pseudocarcharidae-Odontaspididae-Megachasmidae group. On the contrary, Alopiidae was recovered as monophyletic and sister taxon of the Megachasmidae-Pseudocarcharidae group in Amaral et al. [77]. Our study supports the monophyly of Lamnidae as shown in previous phylogenetic studies [15,24,33,77]. The genus Lamna was placed as a sister taxon to the most nested taxa of *Carcharodon* and *Isurus*, a topology supported by both morphological [94] and molecular data [15,24,33,77]. Moreover, previous morphological and molecular studies [28,95] placed Mitsukuridae basal to all other Lamniformes similarly to our findings. However, in Amaral et al. [77], Mitsukurinidae was placed either as a basal sister taxon to the Alopiidae-Megachasmidae-Pseudocarcharidae group in the ML tree topology or as basal taxon to all Lamniformes in the BI tree topology. According to the Bayesian mitogenomic phylogeny (Figure 1a), Carcharhinifromes was represented by seven families, with Carcharhinidae showing paraphyly and Scyliorhinidae placed at the most basal position of this order, similarly to previous molecular studies [15,24,33,77]. Scyliorhinidae was followed by Proscylliidae, which was sequenced by Pentanchidae and later by Triakidae and Hemigaleidae, with a grouping between Sphyrnidae and Carcharhinidae in the most nested position. The embedment of Sphyrnidae within Carcharhiniformes was also supported by Vélez-Zuazo and Agnarsson [33] based on nuclear and mitochondrial genes, and by Da Cunha et al. [15] based on complete mitogenomic analysis. Our results enhance previous morphological studies in which Scyliorhinidae represented the basal lineage followed by Triakidae and the clade formed by the derived Sphyrnidae and Carcharhinidae [88,92]. The phylogenetic placement of all families with Carcharhiniformes was supported by high posterior probability values. The only exception was in the case of Sphyrnidae (represented by Sphyrna and Eusphyrna species) that was grouped within Carcharhinidae (represented by Carcharhinus, Glyphis, Lamniopsis, Prionace and Triaenodon species) with 0.64 probability. A second group of Carcharhinidae was represented by Loxodon macrorhinus basal to the Rhizoprionodon-Scoliodon group, and it was recovered following Galeocerdo cuvier with high posterior probability value (1.0). Similarly, to our results, Vélez-Zuazo and Agnarsson [33] assigned Scoliodon as the sister genus of Rhizoprionodon but no Loxodon species was included in their analysis, while Naylor et al. [96] assigned *Scoliodon* deeply nested within the Carcharhinidae, as the sister genus of *Loxodon* in a group with Rhizoprionodon as the basal genus. Paraphyly within Carcharhinidae is commonly observed in phylogenetic studies with differences attributed to the selected genes or the number of species included in the analysis [33,77,96]. Actually, lack of monophyly has been observed in almost all of the families within Carcharhiniformes, in a variety of studies using a different combination of molecular markers, most of them from the mtDNA [32,33,97]. In the present study, most of Carcharhinus species were placed within Carcharhinidae with medium probability values based on BI analysis but showed polytomy in ML analysis (Figures 1a and 2a). A similar pattern of polytomy within Carcharhinidae has previously been observed [15] and related to the low levels of intrinsic genetic variability of sharks [98].

The Batoidea were split to four monophyletic orders. Based on the BI analysis, a basal division was found between a first group recovered with medium support (0.71) and formed by Rajiformes placed as a sister taxon to Torpediniformes, and a second group recovered with strong support (1.00) and formed by Rhinopristiformes placed as a sister taxon to Myliobatiformes (Figure 1b). Rajiformes were placed as a sister taxon to Torpediniformes, similarly to the results Da Cunha [15] and Amaral et al. [77]. A low support regarding the division of Torpediniformes from other batoids was also recovered in Amaral et al. [77] where only Narcine entemedor was included in the analysis, but also in Gaitán-Espitia et al. [99] presenting the most complete phylogeny of Torpediniformes based on 11 PCGs and including 6 species from the genera Torpedo, Typhlonarke and Narcine. It is worth mentioning that based on the ML analysis, Torpediniformes was placed with low support (46) as a basal taxon to the Rhinopristiformes-Myliobatiformes group, and all these three orders were placed as a sister group to Rajiformes (Figure 2b). According to the Bayesian mitogenomic phylogeny (Figure 1b), Rajiformes was represented by three families, the basal Anacanthobatidae, Arhynchobatidae and Rajidae. Within Rajiformes, all families formed monophyletic groups with species of the genus Bathyraja (Arhynchobatidae) being involved in a large polytomy. Torpediniformes was represented by two families, the Torpedinidae, represented by Torpedo marmorata, which was placed as a sister and basal taxon to Narcinidae represented by three Narcine species. Rhinopristiformes was represented by four families with Zapteryx exasperata (Rhinobatidae) placed at the most basal position of the order and followed by a group formed by two *Rhinobatos* species. Following this, the monophyletic Pristidae was placed as a sister taxon to the Rhinobatidae-Rhinidae-Rhynchobatidae group. Finally, Myliobatiformes was the most derived phylogenetically order of the batoids, represented by five families with Aetobatus *flagellum* (Myliobatidae) placed at the most basal position. The topology of all families within Myliobatiformes was highly supported with only Myliobatidae showing paraphyly. Dasyatidae was recovered as sister family to Potamotrygonidae similarly with Amaral et al. [77]. Finally, Gymnuridae was recovered as a sister taxon to Pleisiobatidae, and both families were more closely related to Myliobatidae than Dasyatidae.

## 4. Conclusions

Elasmobranchs consist one of the most diverse groups, represented by almost 1200 taxonomically valid species, and at the same time are among the most vulnerable taxa to exploitation [23]. The present study describes analytically for the first time the complete mitogenome of nine elasmobranchs, namely Bathytoshia centroura, Dasyatis tortonesei, Galeus melastomus, Gymnura altavela, Odontaspis ferox, Prionace glauca, Raja undulata, Squalus blainville and Torpedo marmorata. The phylogenetic placement of these species among modern selachians was highly supported based on both ML and BI phylogenetic analyses, also showing an effect of their reproductive mode. The phylogenetic tree reconstructions confirmed the monophyly of Neoselachii and Batoidea similarly to the most recent elasmobranch phylogenies. The tree topologies supported the division of Neoselachii to Squalomorphii and Galeomorphii, as well as the monophyly of the eight shark orders. Differences found regarding the phylogenetic placement at family or species level among modern elasmobranch phylogenies were mainly related to the variation in taxonomic sampling. This highlights the need to target taxonomic sampling in particular regions of the topology by prioritizing especially threatened species that embody significant amounts of unique evolutionary history [2]. As mitogenomic data from different taxa become available, evolutionary questions concerning Chondrichthyes are likely to be answered. In this respect, present findings contribute towards a more comprehensive understanding of the relationships among elasmobranchs and establishing conservation priorities, given that information about species' evolutionary history and the status of their close relatives, can impact conservation planning, especially for those species already identified as being under some level of threat.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4425/12/3/324/s1, Figure S1: Representative map of the complete mitochondrial genome of Bathytoshia centroura (Accession Number: MT274568); Figure S2: Representative map of the complete mitochondrial genome of Dasyatis tortonesei (Accession Number: MT274569); Figure S3: Representative map of the complete mitochondrial genome of Galeus melastomus (Accession Number: MT274570); Figure S4: Representative map of the complete mitochondrial genome of Gymnura altavela (Accession Number: MT274571); Figure S5: Representative map of the complete mitochondrial genome of Odontaspis ferox (Accession Number: MT274572); Figure S6: Representative map of the complete mitochondrial genome of Prionace glauca (Accession Number: MT274573); Figure S7: Representative map of the complete mitochondrial genome of Raja undulata (Accession Number: MT274574); Figure S8: Representative map of the complete mitochondrial genome of Squalus blainville (Accession Number: MT274575); Figure S9: Representative map of the complete mitochondrial genome of Torpedo marmorata (Accession Number: MT274576); Figure S10: Secondary structure model of the tRNASer (AGY) gene generated in MITOS webserver of the nine elasmobranchs included in the present study; Table S1: List of the complete mitogenomes included in the present study. The assembled mitogenomes are highlighted in grey and the reference mitogenomes in bold characters. The taxonomy and IUCN status of each species is also presented; Table S2: Substitution models selected by JModelTest v2.1.7 for each PCG and overall; Table S3: Organization of the complete mitogenome of the nine elasmobranchs included in the present study; Table S4: Base composition (%) in the complete mitogenome and PCGs of the nine elasmobranchs included in the present study; Table S5: Codon usage of the PCGs of the mitogenomes of the nine elasmobranchs included in the present study; Table S6: Description of the tandem repeats found in the control region of the assembled mitogenomes.

**Author Contributions:** Conceptualization: M.R. and V.K.; experimental part: V.K.; methodology: M.R. and V.K.; sample acquisition: V.K.; formal analysis: V.K., M.R., S.M. and K.V.; bibliographic investigation: V.K.; resources: M.R.; data curation: V.K. and M.R.; writing—original draft preparation: V.K.; review & contribution to the paper's final form: V.K., M.R.; supervision: M.R.; funding acquisition: M.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

**Institutional Review Board Statement:** All specimens were incidentally caught by commercial trawlers or long-liners in the Aegean Sea, Greece and were kindly provided by fishermen. The experimental design was performed by an accredited researcher (MR: CZ03540) and was approved by the ethical committee of the Faculty of Science, Charles University, Czech Republic (UKPRF/28830/2021).

Informed Consent Statement: Not applicable.

**Data Availability Statement:** The obtained complete mitogenomes were deposited in GenBank under the accession numbers MT274568–MT274575.

Acknowledgments: We deeply thank Luciano Gomes Fischer (Instituto de Biodiversidade e Sustentabilidade, NUPEM/UFRJ, Rio de Janeiro, Brazil) for giving us the permission to use the photograph of *Bathytoshia centroura*. The central fish market in Athens (Greece) is acknowledged for kindly providing the tissue samples. Computational resources were supplied by the project "e-Infrastruktura CZ" (e-INFRA LM2018140) provided within the program Projects of Large Research, Development and Innovations Infrastructures.

Conflicts of Interest: The authors declare no conflict of interest.

## References

- 1. Raup, D.; Sepkoski, J. Mass extinctions in the marine fossil record. *Science* **1982**, *215*, 1501–1503.
- Stein, R.W.; Mull, C.G.; Kuhn, T.S.; Aschliman, N.C.; Davidson, L.N.K.; Joy, J.B.; Smith, G.J.; Dulvy, N.K.; Mooers, A.O. Global priorities for conserving the evolutionary history of sharks, rays and chimaeras. *Nat. Ecol. Evol.* 2018, *2*, 288–298.
- Grogan, E.; Lund, R. The origin and relationships of early Chondrichthyes. In *Biology of Sharks and their Relatives*; Carrier, J., Musick, J., Heithaus, M., Eds.; CRC Press: Washington, DC, USA, 2004; pp. 3–31.
- Musick, J.A.; Ellis, J.K. Reproductive evolution of chondrichthyans. In *Reproductive Biology and Phylogeny of Chondrichthyes:* Sharks, Rays and Chimaeras; Hamlett, W.C., Ed.; Science Publishers: Endfield, 2005; pp. 1–10.
- 5. Kousteni, V.; Megalofonou, P. Aging and life history traits of the longnose spiny dogfish in the Mediterranean: New insights into conservation and management needs. *Fish. Res.* **2015**, *168*, 6–19.

- 6. Kousteni, V.; Megalofonou, P. Reproductive strategy of *Scyliorhinus canicula* (L., 1758): A holistic approach based on macroscopic measurements and microscopic observations of the reproductive organs. *Mar. Freshwater Res.* **2019**, *71*, 596–616.
- Metochis, C.P.; Carmona-Antoñanzas, G.; Kousteni, V.; Damalas, D.; Megalophonou, P. Population structure and aspects of the reproductive biology of the blackmouth catshark, *Galeus melastomus* Rafinesque, 1810 (Chondrichthyes: Scyliorhinidae) caught accidentally off the Greek coasts. J. Mar. Biolog. Assoc. U.K. 2016, 98, 909–925.
- Heinicke, M.P.; Naylor, G.J.P.; Hedges, S.B. Cartilaginous fishes (Chondrichthyes). In *The Time Tree of Life*; Hedges, S.B., Kumar, S., Eds.; Oxford University Press: New York, NY, USA, 2009; p. 320.
- 9. Ebert, D.A.; Fowler, S.L.; Compagno, L.J.V. Sharks of the World: A Fully Illustrated Guide; Wild Nature Press: Plymouth, UK, 2013.
- 10. Weigmann, S. Annotated checklist of the living sharks, batoids and chimaeras (Chondrichthyes) of the world, with a focus on biogeographical diversity. *J. Fish Biol.* **2016**, *88*, 837–1037.
- 11. Wetherbee, B.M.; Cortés, E. Food consumption and feeding habits. In *Biology of Sharks and Their Relatives*; Carrier, J.C., Musick, J.A., Heithaus, M.R., Eds.; CRC Press: Boca Raton, FL, USA, 2004; pp. 223–244.
- 12. Kousteni, V.; Karachle, P.K.; Megalofonou, P. Diet and trophic level of the longnose spurdog *Squalus blainville* (Risso, 1826) in the deep waters of the Aegean Sea. *Deep Sea Res. Part I* 2017, *124*, 93–102.
- 13. Kousteni, V.; Karachle, P.K.; Megalofonou, P. Diet of the small-spotted catshark *Scyliorhinus canicula* in the Aegean Sea (eastern Mediterranean), *Mar. Biol. Res.* 2017, *13*, 161–173.
- 14. Matich, P.; Heithaus, M.R.; Layman, C.A. Contrasting patterns of individual specialization and trophic coupling in two marine apex predators. *J. Anim. Ecol.* **2011**, *80*, 294–305.
- Da Cunha, D.B; da Silva Rodrigues-Filho, L.F.; de Luna Sales, J.B. A review of the mitogenomic phylogeny of the Chondrichthyes. In *Chondrichthyes-Multidisciplinary Approach*; Da Cunha, D.B., da Silva Rodrigues-Filho, L.F., de Luna Sales, J.B., Eds.; TechOpen: 2017; pp. 114–126.
- Venkatesh, B.; Kirkness, E.F.; Loh, Y.H.; Halpern, A.L.; Lee, A.P.; Johnson, J.; Dandona, N.; Viswanathan, L.D.; Tay, A.; Venter, J.C.; et al. Survey sequencing and comparative analysis of the elephant shark (*Callorhinchus milii*) genome. *PLoS Biol.* 2007, 5, 932–944.
- Fowler, S.L.; Reed, T.M.; Dipper, F.A. *Elasmobranch Biodiversity, Conservation and Management*; IUCN Species Survival Commission Shark Specialist Group: Gland, Switzerland; Cambridge, UK, 2002.
- Clarke, S.C.; McAllister, M.K.; Milner-Gulland, E.J.; Kirkwood, G.P.; Michielsens, C.G.J.; Agnew, D.J.; Pikitch, E.K.; Nakano, H.; Shivji, M.S. Global estimates of shark catches using trade records from commercial markets. *Ecol. Lett.* 2006, *9*, 1115–1126.
- Stevens, J.D.; Bonfil, R.; Dulvy, N.K.; Walker, P. The effects of fishing on sharks, rays and chimaeras (chondrichthyans), and the implications for marine ecosystems. *ICES J. Mar. Sci.* 2000, *57*, 476–494.
- 20. Dudley, S.F.J.; Simpfendorfer, C.A. Population status of 14 shark species caught in the protective gillnets off KwaZulu-Natal beaches, South Africa, 1978-2003. *Mar. Freshw. Res.* **2006**, *57*, 225–240.
- 21. Ferretti, F.; Worm, B.; Britten, G.L.; Heithaus, M.R.; Lotze, H.K. Patterns and ecosystem consequences of shark declines in the ocean. *Ecol. Lett.* **2010**, *13*, 1055–1071.
- 22. Myers, R.A.; Worm, B. Extinction, survival or recovery of large predatory fishes. Phil. Trans. R. Soc. Lond. B. 2005, 360, 13–20.
- Dulvy, N.K.; Fowler, S.L.; Musick, K.A.; Cavanagh, R.D.; Kyne, P.M.; Harrison, L.R.; Carlson, J.K.; Davidson, L.N.K.; Fordham, S.V.; Francis, M.P.; et al. Extinction risk and conservation of the world's sharks and rays. *eLife* 2014, 3, e00590.
- Díaz-Jaimes, P.; Bayona-Vásquez, N.J.; Adams, D.H.; Uribe-Alcocer, M. Complete mitochondrial DNA genome of bonnethead shark, *Sphyrna tiburo*, and phylogenetic relationships among main superorders of modern elasmobranchs. *Meta Gene* 2016, 7, 48–55.
- Iglésias, S.P.; Toulhout, L.; Sellos, D.P. Taxonomic confusion and market mislabelling of threatened skates: Important consequences for their conservation status. *Aquat. Conserv.* 2010, 20, 319–333.
- 26. White, W.T.; Last, P.R. A review of the taxonomy of chondrichthyan fishes: A modern perspective. J. Fish Biol. 2012, 80, 901–917.
- 27. Shirai, S. Phylogenetic relationships of the angel sharks, with comments on elasmobranch phylogeny (Chondrichthyes, Squatinidae). *Copeia* **1992**, *2*, 505–518.
- Shirai, S. Phylogenetic interrelationships of neoselachians (Chondrichthyes: Euselachii). In *Interrelationships of Fishes*; Stiassny, M.L.J., Parenti, L.R., Johnson, G.D., Eds.; Academic Press: San Diego, CA, USA, 1996; pp. 9–34.
- 29. Carvalho, M.R. Higher-level elasmobranch phylogeny, basal squaleans, and paraphyly. In *Interrelationships of Fishes*; Stiassny, M.L.J., Parenti, L.R., Johnson, G.D., Eds.; Academic Press: London, UK, 1996; pp. 35–62.
- Carvalho, M.R.; Maisey, J.G. Phylogenetic relationships of the late jurassic shark *Protospinax* Woodward 1919 (Chondrichthyes: Elasmobranchii). In *Mesozoic Fishes: Systematics and Paleoecology*; Arratia, G., Viohl, G., Eds.; Verlag Dr Friedrich Pfeil: Munich, Germany, 1996; pp. 9–46.
- 31. Douady, C.J.; Dosay, M.; Shivji, M.S.; Stanhope, M.J. Molecular phylogenetic evidence refuting the hypothesis of Batoidea (rays and skates) as derived sharks. *Mol. Phylogenet. Evol.* **2003**, *26*, 215–221.
- Human, B.A.; Owen, E.P.; Compagno, L.J.V.; Harley, E.H. Testing morphologically based phylogenetic theories within the cartilaginous fishes with molecular data, with special reference to the catshark family (Chondrichthyes; Scyliorhinidae) and the interrelationships within them. *Mol. Phylogenet. Evol.* 2006, *39*, 384–391.
- Vélez-Zuazo, X.; Agnarsson, I. Shark tales: A molecular species-level phylogeny of sharks (Selachimorpha, Chondrichthyes). Mol. Phylogenet. Evol. 2011, 58, 207–217.

- Naylor, G.J.P.; Ryburn, J.A.; Fedrigo, O.; López, J.A. Phylogenetic relationships among the major lineages of modern elasmobranchs. In *Reproductive Biology and Phylogeny*; Hamlett, W.C., Jamieson, B.G.M., Eds.; Science Publishers: 2005; pp. 1–25.
- 35. Kousteni, V.; Bakiu, R.; Benhmida, A.; Crocetta, F.; Di Martino, V.; Dogrammatzi, A.; Doumpas, N.; Durmishaj, S.; Giovos, I.; Gökoğlu, M.; et al. New Mediterranean Biodiversity Records. *Med. Mar. Sci.* **2019**, *20*, 230–247.
- 36. Kousteni, V.; Kasapidis, P.; Kotoulas, G.; Megalofonou, P. Strong population genetic structure and contrasting demographic histories for the small-spotted catshark (*Scyliorhinus canicula*) in the Mediterranean Sea. *Heredity* **2015**, *114*, 333–343.
- Kousteni, V.; Kasapidis, P.; Kotoulas, G.; Megalofonou, P. Evidence of high genetic connectivity for the longnose spurdog Squalus blainville in the Mediterranean Sea. Med. Mar. Sci. 2016, 17, 371–383.
- Brown, W.M. The mitochondrial genome of animals. In *Molecular Evolutionary Genetics*; MacIntyre, R.D., Ed.; Plenum: New York, NY, USA, 1985; pp. 95–130.
- Chen, X.; Ai, W.; Ye, L.; Wang, X.; Lin, C.; Yang, S. The complete mitochondrial genome of the grey bamboo shark (*Chiloscyllium griseum*) (Orectolobiformes: Hemiscylliidae): Genomic characterization and phylogenetic application. *Acta Oceanol. Sin.* 2013, 32, 59–65.
- 40. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. J. Mol. Biol. 1990, 215, 403–410.
- 41. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120.
- 42. Andrews, S. FastQC: A Quality Control Tool for High Throughput Sequence Data. Available online: http://www.bioinformatics.babraham.ac.uk/projects/fastqc (accessed on 14 November 2019).
- Kearse, M.; Moir, R.; Wilson, A.; Stones-Havas, S.; Cheung, M.; Sturrock, S.; Buxton, S.; Cooper, A.; Markowitz, S.; Duran, C.; et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 2012, 28, 1647–1649.
- 44. Iwasaki, W.; Fukunaga, T.; Isagozawa, R.; Yamada, K.; Maeda, Y.; Satoh, T.P.; Sado, T.; Mabuchi, K.; Takeshima, H.; Miya, M.; et al. MitoFish and MitoAnnotator: A mitochondrial genome database of fish with an accurate and automatic annotation pipe-line. *Mol. Biol. Evol.* 2013, *30*, 2531–2540.
- Lagesen, K.; Hallin, P.F.; Rødland, E.; Stærfeldt, H.H.; Rognes, T.; Ussery, D.W. RNammer: Consistent annotation of rRNA genes in genomic sequences. *Nucleic Acids Res.* 2007, 35, 3100–3108.
- 46. Chan, P.P.; Lowe; T.M. tRNAscan-SE: Searching for tRNA genes in genomic sequences. Methods Mol Biol. 2019, 1962, 1–14.
- 47. Bernt, M.; Donath, A.; Jühling, F.; Externbrink, F.; Florentz, C.; Fritzsch, G.; Pütz, J.; Middendorf, M.; Stadler, P.F. MITOS: Improved de novo metazoan mitochondrial genome annotation. *Mol. Phylogenet. Evol.* **2013**, *69*, 313–319.
- 48. Benson, G. Tandem repeats finder: A program to analyze DNA sequences. Nucleic Acids Res. 1999, 27, 573–580.
- 49. Kumar, S.; Nei, M.; Dudley, J.; Tamura, K. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform*. 2008, *9*, 299–306.
- Perna, N.T.; Kocher, T.D. Patterns of nucleotide composition at fourfold degenerate sites of animal mitochondrial genomes. J. Mol. Evol. 1995, 41, 353–358.
- 51. Stothard, P. The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques* **2000**, *28*, 1102–1104.
- Sharp, P.M.; Tuohy, T.M.F.; Mosurski, K.R. Codon usage in yeast: Cluster analysis clearly differentiates highly and lowly expressed genes. *Nucleic Acids Res.* 1986, 14, 5125–5143.
- Rozas, J.; Ferrer-Mata, A.; Sánchez-DelBarrio, J.C.; Guirao-Rico, S.; Librado, P.; Ramos-Onsins, S.E.; Sánchez-Gracia, A. DnaSP
  DNA sequence polymorphism analysis of large data sets. *Mol. Biol. Evol.* 2017, 34, 3299–3302.
- 54. Higgins, D.G. CLUSTAL V: Multiple alignment of DNA and protein sequences. *Methods Mol. Biol.* 1994, 25, 307–318.
- 55. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and parallel computing. *Nat. Methods* **2012**, *9*, 772–772.
- Guindon, S.; Dufayard, J.F.; Lefort, V.; Anisimova, M.; Hordijk, W.; Gascuel, O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst. Biol. 2010, 59, 307–321.
- Ronquist, F.; Huelsenbeck, J.P. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 2003, 19, 1572– 1574.
- Rambaut, A. FigTree v1.4, A Graphical Viewer of Phylogenetic Trees, 2014. Available online: http://tree.bio.ed.ac.uk/software/figtree/ (accessed on 26/10/2020).
- 59. Vargas Caro, C.A. Skate-ing on thin ice: Molecular Ecology of Longnose Skates in the Southeast Pacific Ocean. PhD Thesis, The University of Queensland, Brisbane, Australia, 2017.
- Inoue, J.G.; Miya, M.; Lam, K.; Tay, B.H.; Danks, J.A.; Bell, J.; Walker, T.I.; Venkatesh, B. Evolutionary origin and phylogeny of the modern holocephalans (Chondrichthyes: Chimaeriformes): A mitogenomic perspective. *Mol. Biol. Evol.* 2010, 27, 2576–2586.
- 61. Boore, J.L. Animal mitochondrial genomes. *Nucleic Acids Res.* **1999**, *27*, 1767–1780.
- Ojala, D.; Montoya, J.; Attardi, G. tRNA punctuation model of RNA processing in human mitochondria. *Nature* 1981, 290, 470– 474.
- 63. Hester, J.; Atwater, K.; Bernard, A.; Francis, M.; Shivji, M.S. The complete mitochondrial genome of the basking shark *Cetorhinus maximus* (Chondrichthyes, Cetorhinidae). *Mitochondr. DNA* **2015**, *26*, 730–731.
- 64. Boore, J.L.; Macey, J.R.; Medina, M. Sequencing and comparing whole mitochondrial genomes of animals. *Methods Enzymol.* **2005**, 395, 311–348.

- 65. Satoh, T.P.; Miya, M.; Mabuchi, K.; Nishida, M. Structure and variation of the mitochondrial genome of fishes. *BMC Genom.* **2016**, *17*, 719.
- 66. Kitamura, T.; Takemura, A.; Watabe, S.; Taniuchi, T.; Shimizu, M. Mitochondrial DNA analysis for the cytochrome *b* gene and D-loop from the bull shark *Carcharhinus leucas*. *Fish. Sci.* **1996**, *62*, 21–27.
- Castro, A.L.F.; Stewart, B.S.; Wilson, S.G.; Hueter, R.E.; Meekan, M.G.; Motta, P.J.; Bowen, B.W.; Karl, S.A. Population genetic structure of Earth's largest fish, the whale shark (*Rhincodon typus*). *Mol. Ecol.* 2007, *16*, 5183–5192.
- 68. Ramírez-Macías, D.; Vázquez-Juárez, R.; Galván-Magaña, F.; Munguía-Vega, A. Variations of the mitochondrial control region sequence in whale sharks (*Rhincodon typus*) from the Gulf of California, Mexico. *Fish. Res.* **2007**, *84*, 87–95.
- 69. Lee, W.J.; Conroy, J.; Howell, W.H.; Kocher, T.D. Structure and evolution of teleost mitochondrial control region. *J. Mol. Evol.* **1995**, *41*, 54–66.
- Brown, J.R.; Beckenbach, K.; Beckenbach, A.T.; Smith, M.J. Length variation, heteroplasmy and sequence divergence in the mitochondrial DNA of four species of sturgeon (*Acipenser*). *Genetics* 1996, 142, 525–535.
- 71. Hoarau, G.; Rijnsdorp, A.D.; Van Der Veer, H.W.; Stam, W.T.; Olsen, J.L. Population structure of plaice (*Pleuronectes platessa* L.) in northern Europe: Microsatellites revealed large scale spatial and temporal homogeneity. *Mol. Ecol.* **2002**, *11*, 1165–1176.
- 72. Maisey, J.G. Higher elasmobranch phylogeny and biostratigraphy. Zool. J. Linn. Soc. 1984, 82, 33–54.
- Bigelow, H.W.; Schroeder, W.C. Sharks. In Fishes of Western North Atlantic, Part 1; Tee-Van, J., Breder, C.M., Hildebrand, S.F., Parr, A.E., Schroeder, W.C., Eds.; Yale University: New Haven, CT, USA, 1948; pp. 59–576.
- Bigelow, H.W.; Schroeder, W.C. Sawfishes, guitarfishes, skates, and rays. In *Fishes of Western North Atlantic, Part 2*; Tee-Van, J., Breder, C.M., Hildebrand, S.F., Parr, A.E., Schroeder, W.C., Eds.; Yale University: New Haven, CT, USA, 1953; pp. 1–514.
- 75. Winchell, C.J.; Martin, AP.; Mallatt, J. Phylogeny of elasmobranchs based on LSU and SSU ribosomal RNA genes. *Mol. Phylogenet. Evol.* **2004**, *31*, 214–224.
- Pavan-Kumar, A.; Gireesh-Babu, P.; Babu, P.P.; Jaiswar, A.K.; Hari Krishna, V.; Prasasd, K.P. Molecular phylogeny of elasmobranchs inferred from mitochondrial and nuclear markers. *Mol. Biol. Rep.* 2014, 41, 447–457.
- 77. Amaral, C.R.L.; Pereira, F.; Silva, D.A.; Amorim, A.; de Carvalho, E.F. The mitogenomic phylogeny of the Elasmobranchii (Chondrichthyes). *Mitochondrial DNA A DNA Mapp. Seq. Anal.* **2018**, *29*, 867–878.
- 78. Gilmore, R.G. Reproductive biology of lamnoid sharks. *Environ. Biol. Fishes.* **1993**, *38*, 95–114.
- 79. Alcock, A. On the utero-gestation in *Trygon bleekeri*. Ann. Mag. Nat. Hist. **1892**, 9, 417–427.
- Dulvy, N.K.; Reynolds, J.D. Evolutionary transitions among egg-laying, live-bearing and maternal inputs in sharks and rays. Proc. R. Soc. B Biol. Sci. 1997, 264, 1309–1315.
- Rolim, F.A.; Rotundo, M.M.; Vaske-Júnior, T. Notes on the reproductive biology of the Brazilian electric ray Narcine brasiliensis (Elasmobranchii: Narcinidae). J. Fish Biol. 2015, 89, 1105–1111.
- 82. Hull, K.L.; Maduna, S.N.; Bester-van der Merwe, A.E. Characterization of the complete mitochondrial genome of the common smoothhound shark, *Mustelus mustelus* (Carcharhiniformes: Triakidae). *Mitochondrial DNA Part B* **2018**, *3*, 962–963.
- Teshima, K.; Koga, S. Studies on sharks. V. Taxonomic characteristics of reproductive organs in Japanese Mustelus. Mar. Biol. 1973, 23, 337–341.
- Smale, M.J.; Compagno, L.J.V. Life history and diet of two southern African smoothhound sharks, *Mustelus mustelus* (Linnaeus, 1758) and *Mustelus palumbes* Smith, 1957 (Pisces: Triakidae). *South Afr. J. Mar. Sci.* 1997, 18, 229–248.
- 85. Wourms, J.P. Reproduction and development in chondrichthyan fishes. *Am. Zool.* **1977**, *17*, 379–410.
- 86. Wourms, J.P.; Lombardi, J. Reflections on the evolution of piscine viviparity. Am. Zool. 1992, 32, 276–293.
- Mallatt, J.; Winchell, C.J. Ribosomal RNA genes and deuterostome phylogeny revisited: More cyclostomes, elasmobranchs, reptiles, and a brittle star. *Mol. Phylogenet. Evol.* 2007, 43, 1005–1022.
- 88. Compagno, L.J.V. Interrelationships of living elasmobranchs. Zoolog. J. Linnean Soc. 1973, 53, 15–61.
- McEachran, J.D.; Dunn, K.A.; Miyake, T. Interrelationships within the batoid fishes (Chondrichthyes: Batoidea). In *Interrelationship of Fishes*; Stiassney, M.L.J., Parenti, L.R., Johnson, G.D., Eds.; Academic Press: New York, NY, USA, 1996; pp. 63-84.
- 90. Compagno, L.J.V. Phylogenetic relationships of living sharks and rays. Am. Zool. 1977, 17, 303–322.
- 91. Regan, C.T. A classification of the selachian fishes. Proc. Zool. Soc. London. 1906, 1906, 722–758.
- 92. White, E.G. Interrelationships of the elasmobranchs with a key to the order Galea. Bull. Am. Mus. Nat. Hist. 1937, 74, 25–138.
- 93. White, E.G. A classification and phylogeny of the elasmobranch fishes. Am. Mus. Novit. 1936, 837, 1–16.
- Compagno, L.J.V. Relationships of the megamouth shark, *Megachasma pelagios* (Lamniformes, Megachasmidae), with comments on its feeding habits. NOAA Tech. Rep. NMFS 1990, 90, 357–379.
- Naylor, G.J.P.; Martin, A.P.; Mattison, E.; Brown, W.M. The inter-relationships of lamniform sharks: Testing phylogenetic hypotheses with sequence data. In *Molecular Systematics of Fishes*; Kocher, T.D., Stepien, C., Eds.; Academic Press: New York, NY, USA, 1997; pp. 199–218.

- Naylor, G.J.P.; Caira, J.N.; Jensen, K.; Rosana, K.A.M.; Straube, N.; Lakner, C. Elasmobranch phylogeny: A mitochondrial estimate based on 595 species. In *Biology of Sharks and Their Relatives*, 2nd ed.; Carrier, J.C.; Musick, J.A., Heithaus, M.R., Eds.; CRC Press: Boca Raton, FL, USA, 2012; pp. 31–56.
- 97. Iglésias, S.P.; Lecointre, G.; Sellos, D.Y. Extensive paraphylies within sharks of the order Carcharhiniformes inferred from nuclear and mitochondrial genes. *Mol. Phylogenet. Evol.* **2005**, *34*, 569–583.
- 98. Rodrigues-Filho, L.F.S.; Rocha, T.C.; Rêgo, P.S.; Schneider, H.; Sampaio, I.; Vallinoto, M. Identification and phylogenetic inferences on stocks of sharks affected by the fishing industry off the Northern coast of Brazil. *Genet. Mol. Biol.* **2009**, *32*, 405–413.
- 99. Gaitáan-Espitia, J.D.; Solano-Iguaran, J.J.; Tejada-Martinez, D.; Quintero-Galvis, J.F. Mitogenomics of electric rays: Evolutionary considerations within Torpediniformes (Batoidea; Chondrichthyes). *Zoolog. J. Linnean Soc.* **2016**, *178*, 257–266.