Black Sea Journal of Engineering and Science
doi: 10.34248/bsengineering.1098258

Open Access Journal
e-ISSN: 2619 – 8991

Research Article
Volume 5 - Issue 3: 116-123 / July 2022

PHYLOGENETIC RELATIONSHIP WITHIN THE GENUS CARCHARHINUS ON THE BASIS OF LEMON SHARK (N. BREVIROSTRIS) POSITION

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Abstract: Many doubts have not been answered about the phylogenetic relationship of the sharks. The morphological models and molecular studies, frequently used these days, can put some species into different order or suborder. The sharks contain about 1% of all fishes, separated into 8 orders. Within these, the largest group is the genus *Carcharhinus*, which includes economically important sharks. A lot of different analyses were done to determine the relationship among these genera. Most of them indicate that phylogenetic relationships at most taxonomic levels remain mysterious for this genera. This study was applied to determine the interrelationship between *Carcharhinus* and *Negaprion* genera based on the lemon shark position and to find out the possible paraphyletic situation of genus *Carcharhinus*, via using ribosomal ITS2 region and mtDNA D-loop for comparison and to get more reliable findings. As a result, based on the ribosomal ITS2 analyses, the lemon shark is placed within the genus *Carcharhinus*, on the other hand, the lemon shark finds a place outside of the genus *Carcarhinus* according to the mtDNA D-loop analyses results. Different findings regarding the position of the lemon shark indicate that it is necessary for more accurate results of the study by using more samples and more gene data.

Keywords: *Carcharhinus*, Evolution, Interrelationship, *N. brevirostris*, Systematics

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Received: April 04, 2022
Accepted: May 30, 2022
Published: July 01, 2022

Cite as: Dosay-Akbulut M. 2022. Phylogenetic relationship within the genus *Carcharhinus* on the basis of lemon shark (*N. brevirostris*) position. BSJ Eng Sci, 5(3): 116-123.

1. Introduction

There are 350 species of shark within Chondrichtyes class. Other known as Cartilaginous fishes include batoid elasmobranchs and Holocephalii as well. Their body was made from cartilaginous tissue instead of bone. But this cartilaginous tissue was supported in someplace with calcium, resembled bone tissue.

The members of this group developed different mechanism, that adapts their body against to the negativity, especially a rich fossil record of them dated back more than 400 million years, providing a lot of information for phylogenetic hypotheses, reached today. This information can be used as an information source of the Chondrichtyans, fishes, and in more general vertebrates' development (Castro, 1983).

The morphological similarities between the shark and batoid elasmobranchs are more than their closeness to the Holocephalii. Especially the Squatiniformes order sharks have similarities with batoids based on their structural similarities. But the classification of these 3 groups (sharks, batoid elasmobranchs, and holocephalii) within and between can change and give different results according to the different criteria. The classification model, that accepted and always gives the same result, is not applied to the practice. The morphological models and molecular studies, frequently used these days, can put some species into different order or suborder (Maisey, 1984).

Members of cartilaginous fish ensure continuity in their species by increasing their resistance to factors that may cause fatigue, as seen in dinosaurs. Taking advantage of their specially shaped upper jaws and teeth, they can be very competent hunters, and with their advanced sensory systems and brains, they can successfully survive attacks from other predators.

All these indicate that they adapted successfully against life's selector characteristics (Compagno, 1990; Tricas, 1997; Taylor, 1997).

So far, about evolution in vertebrates, most of the work had been done mostly in mammals. It cannot forget that there are about 40000 extant species of vertebrates and about 10000 of them are fishes (Martin, 1992).

The sharks contain about 1% of all fishes, separated into 8 orders as 1-Squatiniformes, 2-Pristiophoriformes, 3-Squaliformes, 4-Heterodontiformes, 5-Hexaniformes, 6-Lamniformes, 7- Orectolobiformes, 8- Carcharhiniformes respectively (5). The largest order is Carharhiniformes with 200 species, consist almost 55% of all shark species. Family Carcharhinidae: known as Requem sharks, which is the largest family in this order, includes 50 species

with division into 12 genera. They are *Galeocerdo, Glyphis, Isogomphodon, Lamiopsis Loxodon, Nasolamia, Negaprion, Prionace, Rhizoprionodon, Scoliodon, Triaenodon*, and the largest genus *Carcharhinus*, with 30 species of them belong to it (Mcdiarmid, 1996).

Carcharhiniform sharks, like other sharks, have a very well preserved fossil tooth record. According to these records, the first identification took place in the lower Eocene for Rhizoprionodone and in the lower Miocene for Sphyrna. It has been shown that the differentiation between *Sphyrna*, *Carcharhinus*, and *Negaprion* occurred around 38 Mya at the end of the Eocene (Naylor, 1989; Naylor, 1992).

Different hypotheses were suggested based on some different genus possible inclusion to genus *Carcharhinus* and determine the real interrelationship with genus *Carcharhinus* and others. Some of them indicate a possible sister position of the lemon shark (*N. brevirostris*) to genus *Carcharhinus*.

For example; Cytochrome oxidase I (COI) gene sequences were used to get a more correct answer to the phylogenetic relationship between genus *Carcharhinus* and to others. According to COI gene sequence analysis; Prionace and Negaprion; it is placed as a separate genus outside of *Carcharhinus*, while Sphyrna and Triaenodon are classified together with the genus *Carcharhinus*.

In a related study by the same author; 561 bp Cytochrome b sequence data, were obtained from Galeocerdo and 18 additional species of sharks. In all analyses, *C. falciformis* and *Negaprion* grouped together and at the base of the genus *Carcharhinus* (Martin, 1992).

Another study consists mt sequences and the nRAG1 sequences. This molecular phylogenetic study aimed to better understand relationships within Carcharhiniformes. mtDNA data set included complete Valine tRNA and 16S rRNA and partial 12S rRNA genes for 45 species. According to MP tree analysis, calculated from the mtDNA data set; Negaprion was obtained as the sister group of *Carcharhinus*, though; Compagno' suggestion, based on morphologic characters (Compagno, 1988) and Lavery' in his allozyme research (Lavery, 1992) found Negaprion placed within the genus *Carcharhinus* (Iglésias et al., 2005).

ITS region sequences can be used with useful information for the determining of the species origins or the phylogenetic relationships at different levels. Nuclear ribosomal RNA cistron (rDNA) or some parts of it are frequently used in phylogenetic studies. The rRNA genes are in sequential copies in cistron and express a family of genes that have undergone a rapid and harmonious evolution. Particularly the ITS2 region, which is among the parts within this cistron, has a great advantage in terms of being a locus that has a rapid evolution feature in terms of revealing the phylogenetic relationships between closely related species.

On the other hand, mtDNA D-loop, which representing relatively slowly evolving, also was used in this study to get the results with the minimum of the effect of the evolving rate.

Based on the different studies' findings related to lemon shark (*N. brevirostris*) position, this study was designated to reveal the phylogenetic relationship among the species of genus *Carcharhinus*, to find out the possible paraphyletic situation of genus *Carcharhinus* and interrelationship between *Carcharhinus* and *Negaprion* genera based on lemon shark position, via using ribosomal ITS2 region and mt D-loop for comparison and to get more reliable result.

2. Material and Methods

DNA Sources: For ITS2, 17 species were used. 11 of them from genus *Carcharhinus*, 3 species belongs to genus *Prionace*, 1 species from genus *Negaprion*, 1 of them from *Rhizoprionodon* and the last one from genus *Galeocerdo* were amplified. For mtDNA D-loop; 11 species were used. 6 of them from genus *Carcharhinus*, and other 5 of them, 1 each from genus *Prionace*, *Negaprion*, *Rhizoprionodon*, *Sphyrna*, and *Galeocerdo* were amplified (Table 1 and 2).

All species belong to the genus *Carcharhinus*, except Blue shark, Atlantic sharpnose, scalloped hammerhead, Lemon shark and Tiger shark.

Table 1. The used ridge-backed and smooth-backed species listed (Dosay, 2000)

Ridge-backed species	Smooth-backed species
Caribbean reef	Bull
Sandbar	BlacknoseBignose
Finetooth	
Silky	Spinner
Dusky	Smalltail
	Blacktip
	Blue
	Lemon
	Atlantic sharpnose

Table 2. List of species that were used in this study (Compagno, 1984)

Species name	Common name
C. altimus	Bignose
C. brevipinna	Spinner
C. acronotus	Blacknose
C. falciformis	Silky
C. isodon	Finetooth
C. leucas	Bull
C. limbatus	Blacktip
C. obscurus	Dusky
C. plumbeus	Sandbar
C. perezi	Carib. reef
C. porosus	Smalltail
C. R. terraenovae	Atshnose
C. P. glauca	Blue
C. N. brevirostris	Lemon
C.S. lewini	Scalloped hammerhead
C. G. cuvier	Tiger

2.1. DNA extraction, PCR amplification, and sSequencing

DNA extraction was carried out in two methods. One of them is a phenol/water/chloroform method, on the basis of ABI manual DNA extraction kit or QIAamp tissue kit was used from QIAGEN Company. In the first method 0.2 to 0.5 g. tissue; in the second method, where 0.2-1.2 mg DNA was reached with each mg of isolation, 25 mg of tissue was used each time. The finally obtained genomic DNA was kept in refrigerator.

The ribosomal internal transcribed spacer ITS2 and mtDNA D-loop were amplified via using the polymerase chain reaction (PCR) with below indicated primers.

The PCR amplification primers for ribosomal ITS2 region and mt D-loop within this study are shown below;

ITS2F L	CTACGCCTGTCTGAGTGTC
ITS2R H	ATATGCTTAAATTCAGCGGG
D-loop F:	CCACATACTACCCTCATTCC
D-loop R:	GTATATTAAGGGGGAGGGGG

Primers were designed from a fish sequence that was obtained from Genbank. All PCR amplification was completed via using Perkin Elmer DNA Thermal Cycler 480 or PTC-100TM Programmable Thermal Controller (MJ Research, Inc.).

The sequences were entered into the Eyeball sequence editor (Cabot and Beckenbach, 1989). The best alignment with the lowest parsimony score was used in tree construction. Total about, 1400 bp nucleotide for ITS2 and 2000 bp nucleotide for D-loop were aligned.

The analysis were carried out with using neighbor-joining (NJ) and maximum parsimony (MP) methods within the PHYLIP 3.5c (Felsenstein, 1993) and maximum likelihood (DNAML) from PUZZLE (Strimmer and von Haeseler, 1996) quartet-puzzling approach. Both analysis authenticity were tested with using bootstrapping (Felsenstein, 1985) on the basis of 1000 replications of the data. The kimura-2- parameter distance matrix model was applied to Parsimony analysis with a transition / transversion ratio of 2:0 as well as in DNAML analysis, with 10 times randomizing of the input order.

3. Results

This study's molecular data included ribosomal ITS2 and the mitochondrial D-loop region. The sequence alignment of 1590 bp for the ITS2 region from 17 species data and 2068 bp of the mitochondrial D-loop region from 11 species were used in tree construction. In all data Galeocerdo cuvier (tiger shark) was chosen as an outgroup (Table 3 and 4).

Table 3. Maximum likelihood sequence divergence matrix for ribosomal ITS2 region (Dosay, 2000).

Bull	0.0553	0.0440	0.0626	0.0591	0.0617	0.0960	0.0899	0.0819	0.0733	0.0992	0.0790	0.0763	0.1996	0.3125	0.1523	0.2631
Spinner	0.0530	0.0517	0.0491	0.0562	0.0790	0.0751	0.0722	0.0639	0.0817	0.0874	0.0672	0.1886	0.3003	0.1420	0.2458	
Blacknose	0.0494	0.0468	0.0572	0.0765	0.0694	0.0712	0.0643	0.0817	0.0730	0.0650	0.1762	0.2899	0.1455	0.2416		
Blacktip	0.0438	0.0526	0.0683	0.0618	0.0619	0.0558	0.0693	0.0842	0.0525	0.1841	0.2955	0.1291	0.2307			
Bignose	0.0128	0.0438	0.0398	0.0363	0.0426	0.0594	0.0766	0.0367	0.1826	0.2910	0.1251	0.2178				
Sandbar	0.0546	0.0513	0.0406	0.0477	0.0647	0.0823	0.0467	0.1897	0.3005	0.1346	0.2277					
Blue3	0.0174	0.0201	0.0675	0.0839	0.1000	0.0492	0.1999	0.3156	0.1403	0.2407						
Blue2	0.0165	0.0621	0.0781	0.1023	0.0463	0.1992	0.3042	0.1447	0.2432							
Blue1	0.0574	0.0733	0.1022	0.0437	0.2040	0.3052	0.1417	0.2343								
Dusky	0.0597	0.0933	0.0570	0.2032	0.3047	0.1402	0.2453									
Silky	0.1176	0.0706	0.2225	0.3346	0.1645	0.2498										
Lemon	0.0689	0.1978	0.3534	0.1260	0.2583											
Caribreef	0.1803	0.3280	0.1166	0.2294												
Smalltail	0.4291	0.2488	0.3544													
Sharpnose	0.3803	0.3831														
Finetooth	0.2518															
Tiger																

Table 4. Maximum likelihood sequence divergence matrix for mitochondrial D-loop region data (Dosay, 2000)

Sharpnose	0.0743	0.0978	0.0978	0.0899	0.1030	0.1049	0.1488	0.1136	0.1870	0.1663
Smalltail	0.0564	0.0629	0.0585	0.0547	0.0578	0.1172	0.0868	0.1949	0.1514	
Blacknose	0.0520	0.0496	0.0320	0.0417	0.1052	0.0807	0.2053	0.1411		
Blacktip	0.0475	0.0435	0.0487	0.1088	0.0790	0.1977	0.1466			
Caribreef	0.0411	0.0408	0.1047	0.0779	0.1989	0.1434				
Finetooth	0.0333	0.1005	0.0801	0.1936	0.1393					
Spinner	0.0955	0.0736	0.2029	0.1444						
Blue	0.1308	0.2071	0.1798							
Lemon	0.2013	0.1539								
Scalhammer	0.2261									
Tiger										

ITS2 data MP and NJ analysis placed *N. brevirostris* with the genus *Carcharhinus* with 100% and 98% bootstrap value respectively, even classified in a different genus. Lemon shark position within the genus *Carcharhinus* was supported by ITS2 DNAML and transversion analysis also. In almost all ITS data analysis; lemon shark placed with finetooth shark, which both a member of Smooth-backed species, within genus *Carcharhinus* clade (Table 5).

Table 5. Bootstrap support from various analyses for the inclusion of lemon shark within the genus *Carharhinus* (Dosay, 2000)

Phylogenetic loci	Lemon sh as *Carcharhinus*	Lemon outside *Carcharhinus*
ITS2		
MP	100	0
TVMP	99	0
NJ	98	2
PUZZLE	77	0
D-loop		
MP	38	60
TVMP	8	75

Mitochondrial D-loop region The Maximum Likelihood (DNAML) puzzle analysis, puts lemon shark into *Carharhinus* clade with 73% bootstrap value. Other analyses (NJ, MP, and TVMP) also support lemon shark position within the genus *Carcharhinus* with little lower bootstrap support compare to ITS values. But mtDNA D-loop analysis gave different indications than ITS analysis based on lemon shark nearest species. Mostly, smalltail shark was obtained as closest to the lemon shark, which also both a member of Smooth-backed species, according to mt D-loop analysis.

Table 5 of the sequence divergence of the ITS2 region indicates, the sequence divergence between the genus *Carcharhinus* and lemon sharks from genus *Negaprion* the range about 6-12 %, while within the genus *Carcharhinus* range about 4-7.5 %, which is a little high sequences differences were obtained from inside the genus *Carcharhinus*.

Table 5 of the sequence divergence of the mitochondrial D-loop region indicates that the sequence divergence is, between genus *Carcharhinus* and *N. brevirostris* is 7-8 %, while within the genus *Carcharhinus* is about 3-7 %. This means *N. brevirostris* much closer species to the genus *Carcharhinus* and got almost similar divergence value to within *Carcharhinus* species. The data point that; the most divergent species is *S. lewini*.

3.1. Internal Transcribed Spacer 2

The monophyly of the smooth-backed forms (inclusive of lemon), and a possibility of the lemon shark, is a derived carcharhinid, was a quite well-supported result with especially internal transcribed spacer 2 (ITS2) data. But D-loop DNAML puzzle analysis only supports this finding. The other analysis of D-loop; did not give enough bootstrap value for lemon shark position within genus *Carcharhinus*.

Almost all analyses of molecular data agreed that *N. brevirostris* has a position within the genus *Carcharhinus*. Also, the sequence divergence values, supports this place as well.

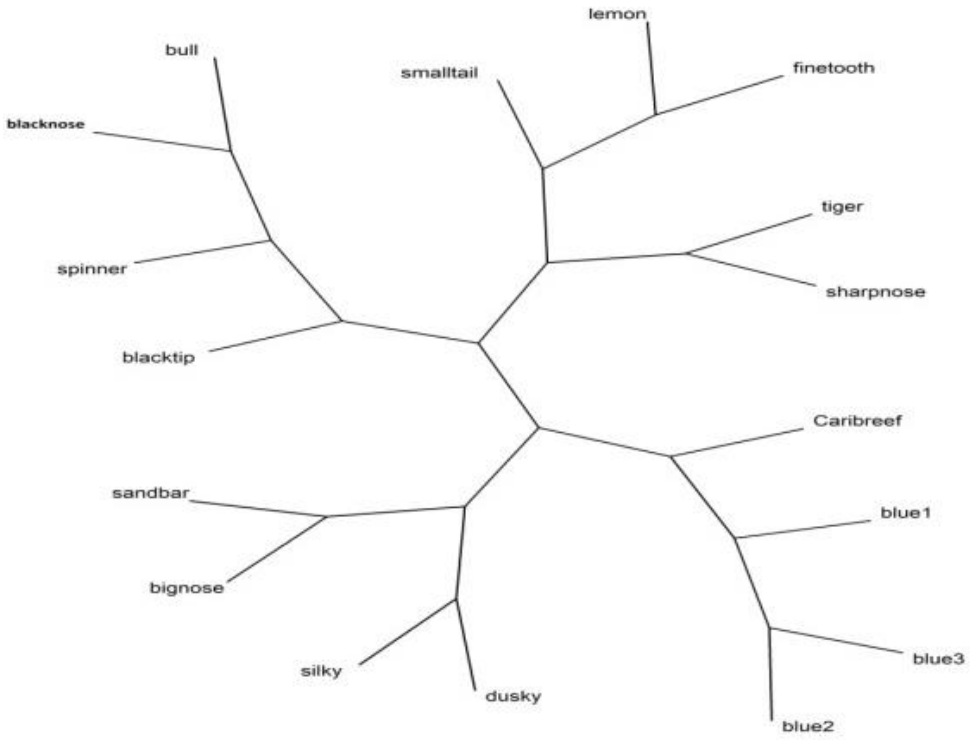

Figure 1. The majority consensus Neigbour Joining (NJ) Bootstrap tree for the ribosomal ITS2 (Dosay, 2000).

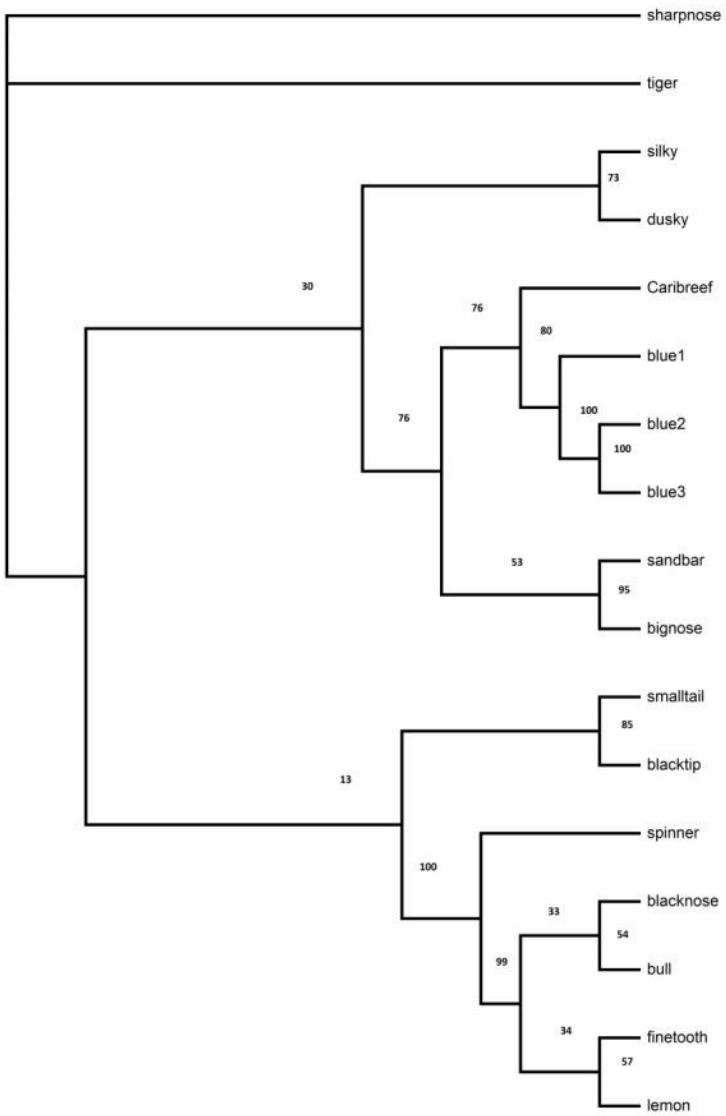

Figure 2. The Majority consensus Transversion Parsimony Bootstrap tree of ribosomal ITS2 (Dosay, 2000).

ITS2 data findings indicate the inclusion of lemon shark inside the carcharhinid clade. This is an arrangement that had been previously suggested by the morphological studies of Compagno (1988) but was not supported in Naylor's allozyme analyses. Support for the inclusion of lemon along with the rest of the carcharhinids is considerable, with 100 and 98% bootstrap support for the monophyly of *Carcharhinus* + lemon, for almost all ITS2 analyses respectively.

Interestingly, maximum likelihood (DNAML) analysis of the ITS2 data splits the ridge-back (+blue) and the smooth-back (inclusive of lemon) into two monophyletic groups.

3.2. D-loop

The other analysis of D-loop; did not give enough bootstrap value for lemon shark position within genus *Carcharhinus*.

4. Discussion and Conclusion

There are uncertainties in the elasmobranchs' classifications and the phylogenetic relationships within and between the class Chondrichthyes. A lot of different studies are carried out to find the relationship within the genus *Carcharhinus*. Different hypotheses were suggested for the possible paraphyletic situation of *Carcharhinus* with the inclusion of lemon shark. *N. brevirostris* position and relation to genus *Carcharhinus* was searched for different criteria.

In the morphological and anatomical comparison; In terms of reproductive characteristics, *Negaprion* and *Carcharhinus* breeds are similar in terms of live birth, matrotropic and having placenta (Dulvy, 1998).

Supporting the findings of this study related to a possible lemon shark position within the genus *Carcharhinus*, Irschick et al. (2017) carried out another study. For comparison; 12 morphometric values and body measurements of 8 different individuals from Carcharhinidae and Ginglymostomatidae families were obtained and used in the calculation of this study. In terms of the general shape of the pectoral fin or the dorsal fin, the first four PC values obtained as a result of the harmonic analysis for each fin were found to be close to each other for these 4 species (lemon, blacktip, nurse and sandbar) (Irschick et al., 2017).

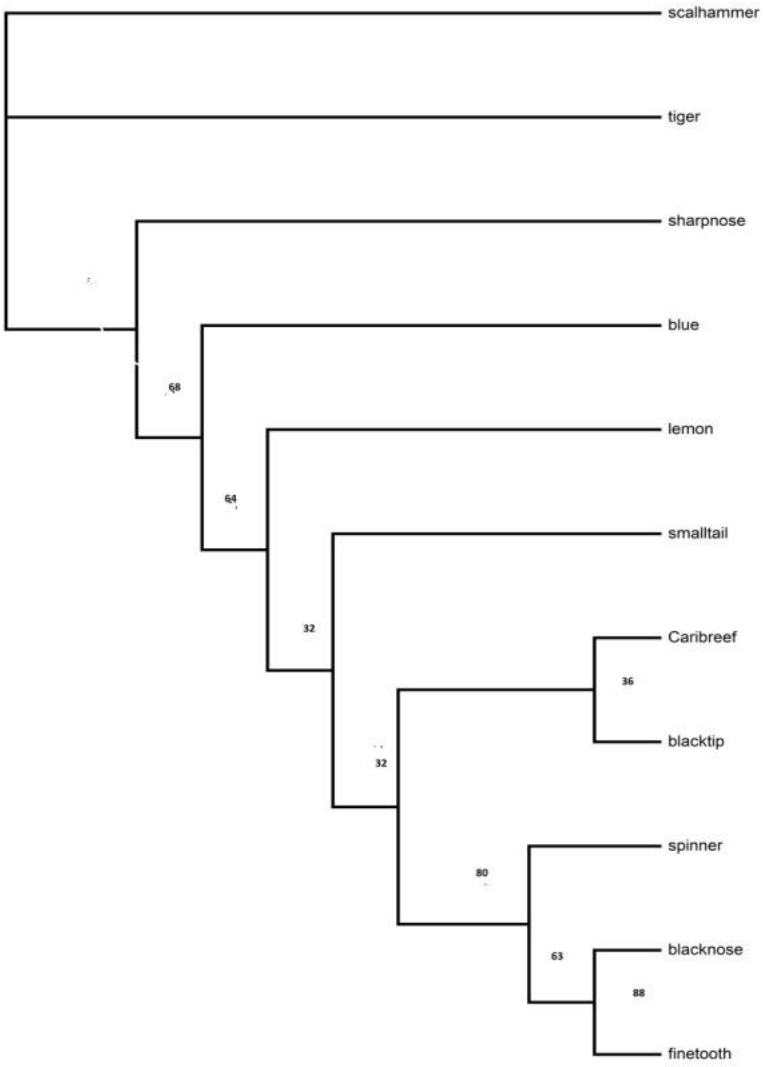

Figure 3. The majority consensus NJ Bootstrap tree for the mtDNA D-loop (Dosay, 2000).

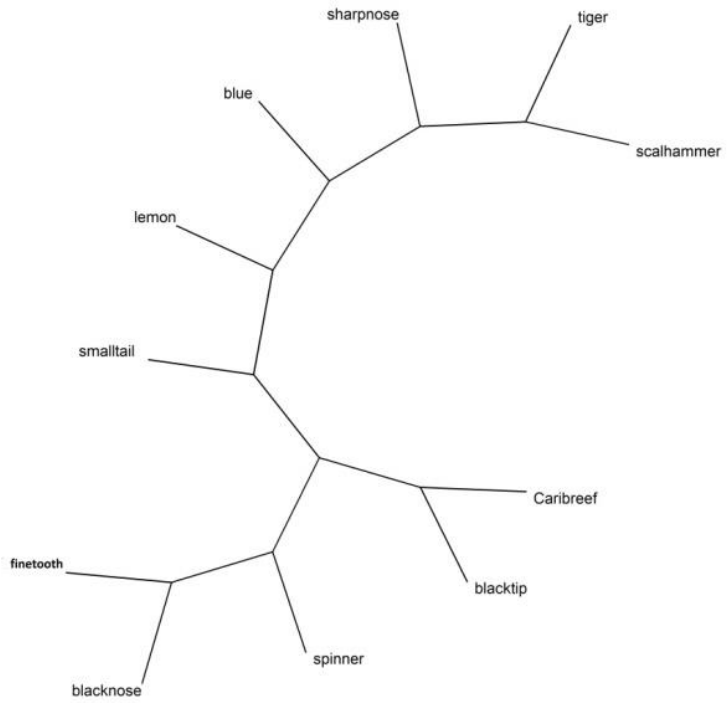

Figure 4. The Maximum Likelihood (DNAML) Tree with branch length of the mtDNA D-loop (Dosay, 2000).

Lavery study based on allozyme data analysis. This study included carcharhinid sharks and 4 related species collected from Australia. The most parsimonious tree was not supported in his study of the genus *Carcharhinus* with monophyly. This genus, as a result of the analysis; It was obtained paraphyletically with the participation of *Negaprion acutudiens and Galecerdo cuvier* from other genera and *Hemipristis elongatus* from different families (Lavery, 1992).

Molecular phylogenetic studies with mtDNA data set and nuclear RAG1 sequences have been recently introduced by Iglésias to reveal relationships within Carcharhiniformes. The mtDNA data set (partial 12S rRNA, full Valine tRNA and 16S rRNA genes) was used in the study. As a result of the analysis, *Negaprion* is the sister group of *Carcharhinus*, whereas Compagno' suggestion according to teeth fossil found *Negaprion* nested within *Carcharhinus* (Iglésias et al., 2005).

In Swift study, transcriptome statistics for nine viviparous shark species were obtained from Atlantic sharpnose shark (*Rhizoprionodon terraenovae*), Blacknose shark (*Carcharhinus acronotus*), Blue shark (*Prionace glauca*), Bull shark (*Carcharhinus leucas*), Caribbean reef shark (*Carcharhinus perezii*), Dusky smoothhound (*Mustelus canis insularis*), Lemon shark (*Negaprion brevirostris*), Sand tiger shark (*Carcharias taurus*) and Tiger shark (*Galeocerdo cuvier*). In the result of 1,197 orthologue alignments' phylogenetic analysis, the lemon shark was placed with bull shark and Blacknose shark from genus *Carcharhinus*, indicating a possible genus *Carcharhinus* paraphyletic situation with lemon shark inclusion (Swift et al., 2016).

Martin's Ph.D. thesis provided some evidence from the perspective of molecular sequence for the possible inclusion of blue shark and lemon shark within the genus *Carcharhinus*. But his other studies carried out with 12S ribosomal gene, cytochrome. b gene and cytochrome oxidase I gene sequence analysis, the lemon shark position and the phylogenetic relationship among the Carcharhinidae sharks were not cleared (Martin, 1992; Martin, 1993; Martin, 1995).

37 species of carcharhiniform sharks were involved to identify protein variation, in Naylor's study. Evolution trees were created with cladistic character and distance Wagner analysis based on these data. In both analyses, Galeocerdo, Sphyrna, Rhizoprionodon, Loxodon, Negaprion and Triaenodon were located outside of *Carcharhinus* (Naylor, 1992).

Different results from different studies show different positions for the place of the lemon shark (*N. brevirostris*) in the classification. Some classify the lemon shark within the genus *Carcharhinus*, while others put it in a position outside of this genus.

Also, our molecular findings gave similar indications, that ribosomal ITS2 all analysis placed lemon shark within the genus *Carcharhinus*, while mtDNA D-loop analysis support mostly lemon shark outside of genus *Carcharhinus*. The lack of congruence in regards to this positioning of lemon shark suggests that a more firm conclusion will have to await further sampling of species and molecular loci.

Author Contributions

All task made by M.D.A. (100%) data acquisition and analysis, writing up, submission and revision. The author reviewed and approved final version of the manuscript.

Conflict of Interest

The author declared that there is no conflict of interest.

Acknowledgements

This study was prepared from corresponding author's PhD thesis.

Ethical Consideration

Ethics committee approval was not required for this study because of there is no animal or human study.

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