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Comparative Histological Analysis of Cerebellum of Representative Species of Elasmobranchii

Análisis Histológico Comparativo del Cerebelo de Especies Representativas de Elasmobranchii

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SUMMARY: In elasmobranch fishes, variations in gross structural organization of cerebellum has been extensively explored. The basic histological features of cerebellum although conserved in the group but the comparative account on subtle cellular variations is largely underestimated. The present study aims to explore the histological and cellular variations in different layers of cerebellar cortex of the representative elasmobranchs' species belonging to different habitat. Our findings showed that the histological architecture of cerebellar granular layer between the examined species varies noticeably. By and large increase cellular density were observed in all the layers of cerebellum in the representative species of shark compared to ray. The findings were then compared and discussed with reference to their habitat and behavior.

KEY WORDS: Elasmobranch; Cerebellum; Evolution; Carcharhinus; Chiloscyllium; Rhinobatos.

INTRODUCTION

Chondrichthyes represents the most evolutionary distinct radiation of vertebrate lineage (Dulvy *et al*., 2021), diverged from the jawed vertebrates around 450 million years ago (MYA) (Hara *et al*., 2018). Over 1100 species of cartilaginous fishes have been described including 611 rays and 536 sharks (Dulvy *et al*., 2021). Around 350 MYA chondrichthyes radiated into Elasmobranchii (5 to 7 pairs of uncovered gill slits) and holocephalii (upper jaw fused to cranium). Elasmobranchii subsequently diversified into number of different species including rays, skates (Batoidea) and sharks (Selachii) (Compagno *et al*., 2005), constituting nearly 96 % of the chondrichthyes species (Compagno, 1977; Yopak *et al*., 2007). Sharks are regarded as living fossils as they are the modern survivors with morphological stasis for 450 million years, since their divergence from vertebrate lineage (Nagalingum *et al*., 2011). Approximately 536 species of sharks are listed in IUCN Red list categories (Dulvy *et al*., 2021), and 79 have been reported to be found along the coastline of Pakistan (Moazzam & Osmany, 2021).

Most of the sharks are pelagic where resources are distributed in open water column nearer to the surface and away from the seabed. This marine habitat supports the extreme migratory and predatory nature of sharks where they are in continuous motion for the search of prey and mates, themselves avoiding the predators (Lisney & Collin, 2006). Pelagic sharks evolved a fusiform body shape with pointed snout and fins, lunate or forked tail, huge red muscle mass and pointed sharp teeth (Bernal *et al*., 2001; Lisney & Collin, 2006). Nevertheless, some sharks and many rays live and feed at the bottom of sea and are referred to as demersal or benthic fishes. They evolved a flat ventral body surface so that they can rest on the substrate. They can protect themselves from predators and remain hidden within seabed sediments. This also favors hunting the prey by camouflaging (Compagno *et al*., 2005; Choo *et al*., 2021). The habitat and behavioral pattern of species is functionally alignable with the anatomical organization of their central nervous system (Nieuwenhuys *et al*., 1998). Hence, the

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study of structural diversity of brain and its major regions provides important insights into the biology and behavioral adaptation of organisms (Lisney & Collin, 2006; Yopak *et al*., 2019).

Given the variations found in the habitat and behavior (related to predation and navigation), anatomical organization of cerebellum holds profound importance in the evolution of coordinating motor signals in elasmobranch species. In elasmobranchs, the cerebellum varies considerably in size and shape, although the basic cerebellar neural circuitry is largely conserved (New, 2001). Cerebellum of all elasmobranch species comprises of an unpaired corpus cerebellum which forms the roof of the metencephalon, and the vestibulo-lateral cerebellum (Montgomery *et al*., 2012; Yopak *et al*., 2020). Histologically, the cerebellum comprises a three-layered cortex and an underlying white matter. The cortex consists of external molecular layer, middle layer/row of Purkinje cells forming direct cerebellar output and an inner granular layer comprising granule cell clusters (Mokhtar, 2020; Yopak *et al*., 2020).

Herein, we have selected three separate species of elasmobranchii namely *Carcharhinus* (shark, pelagic), *Chiloscyllium* (shark, benthic) and *Rhinobatos* (ray, benthic) to explore the histological variation in trilaminar conformation of cerebellum. Findings of the study provide interesting insights to the evolutionary changes in cerebellar microanatomy.

MATERIAL AND METHOD

The study was conducted with the approval of Institutional Review Board for Animal Research and Ethics (# AR.IRB-07/DUHS/Approval/2017/09).

Specie selection and procurement. Three representative species belonging to the pisces were selected. The selected species included guitarfish (*Rhinobatos punctifier*), grey bamboo shark (*Chiloscyllium griseum*) and silky shark (*Carcharhinus falciformis*). Three to six animals of each specie were procured from Karachi fish harbor (24.8490635, 66.9760900). Divergence time of any two species was estimated using Time Tree (Kumar *et al*., 2017).

Radiological examination. At least head of one animal of each specie was subjected to radiological examination, to get plain X-ray films (PA view) in order to ascertain the extent of brain in the cranial cavity (Fig. 1A).

Dissection. The skin from the dorsal surface of the head was removed followed by removal of chips of cartilage of chondrocranium from the tip of the rostrum back to the level of gill slits. The delicate vascular protective membrane called the primitive meninx was given a nick and removed to expose the brain. The attachments of olfactory tract and bulb were severed and brain was lifted gently by severing the attached cranial nerves. All brains were stored in 10 % Buffered Neutral Formalin (BNF) for 2-4 weeks till further processing.

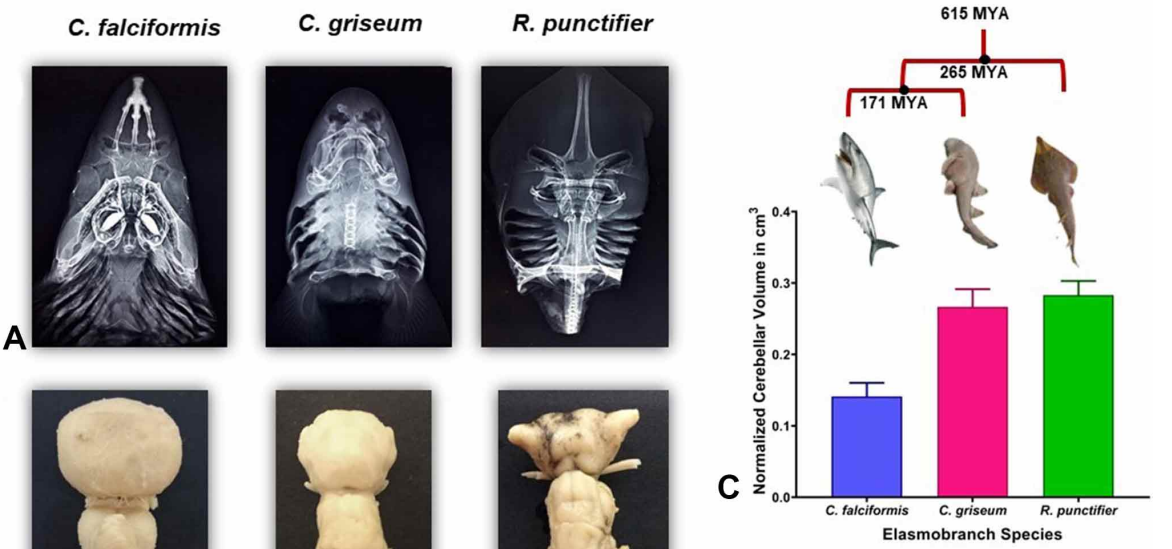

Fig. 1. (A) Radiographic images of skull of representative elasmobranch species, as labeled, (B) Gross anatomy of brain of representative elasmobranch species, where cerebellum of species are shown with red arrows (C) Graph showing comparison of normalized cerebellar volume (NCV) of representative elasmobranch species along with their evolutionary relationship.

Morphometric study. The morphological features of volume of brain and cerebellum were derived from volume displacement method and as volume of ellipsoid, respectively. Volume of ellipsoid was deduced by the following formula:

V= 0.167 x π (3.14159) x length x width x depth (Lisney & Collin, 2006)

All data points were neutralized against brain volume respective to the animal.

Tissue preparation and staining. The brain/cerebellum was cut by a median-sagittal section into two equal halves. The halves were carefully placed into the appropriately labelled tissue cassettes which were then subjected to processing in an automatic tissue processor for dehydration, clearing, infiltration and embedding. Paraffin embedded tissue blocks were subjected to microtomy (5-7 μm). Alternate slides were subjected to conventional hematoxylin and eosin (H&E) staining (Bancroft & Gamble, 2008) and modified formal-thionin block impregnation (Bancroft & Cook, 1994).

Microscopy. The H&E-stained sections were studied on light microscope at 200x and 1600x magnifications to measure the thickness and cellular count (including neurons and neuroglia) of molecular and granular layers in 50μm^2 area. The formal-thionin stained sections were used for neuronal count in molecular, granular and Purkinje layers and for measuring diameter of Purkinje cell. Microscopy was conducted using Leica DM2500 LED and OptikaB3 microscopes. All photomicrographs were imported in Image J v.1.50i for micrometric measurements.

Statistical Analysis. The normality of data distribution was assessed by Kolmogorov-Smirnov test. Statistical significance of the values of normally distributed and skewed data were assessed by student t-test and Mann-Whitney-U test, respectively. Graphical representations of the data were developed using Graph Pad Prism v.8.0.1. In all cases, the p-values of less than 0.05 were considered statistically significant.

RESULTS

The species of elasmobranchs were selected on the basis of their position in the evolutionary tree to represent major lineages of elasmobranchii, such as *C. falciformis*, *C. griseum* and *R.punctifier* belongs to Carcharhinidae, Hemiscylliidae, Rhinobatidae family, respectively. The radiographic images were obtained to ascertain the extent of brain (Fig. 1A). The dissected brain showed cerebellum located dorsally, caudal to telencephalon/cerebrum. Morphologically, the cerebellum appeared ellipsoid with foliations visible externally (Fig. 1B). The Normalized Cerebellar Volume (NCV) was obtained by dividing cerebellar volume against the total brain volume. *R. punctifier* was found to have highest NCV amongst the examined species, while *C. falciformis* showed lowest observed value of the trait. However, no statistical significance was observed between these values (Fig. 1C).

Histological analysis of H&E-stained sections of the cerebella of examined species showed multiple folia with clearly demarcated three primary layers of cerebellar cortex namely Molecular Layer (ML), Purkinje Cell (PC) Layer and Granular Layer (GL). The Purkinje cells form one or two cells thick layer sandwiched between ML and GL. The PC appeared to have large, ovoid or triangular soma with

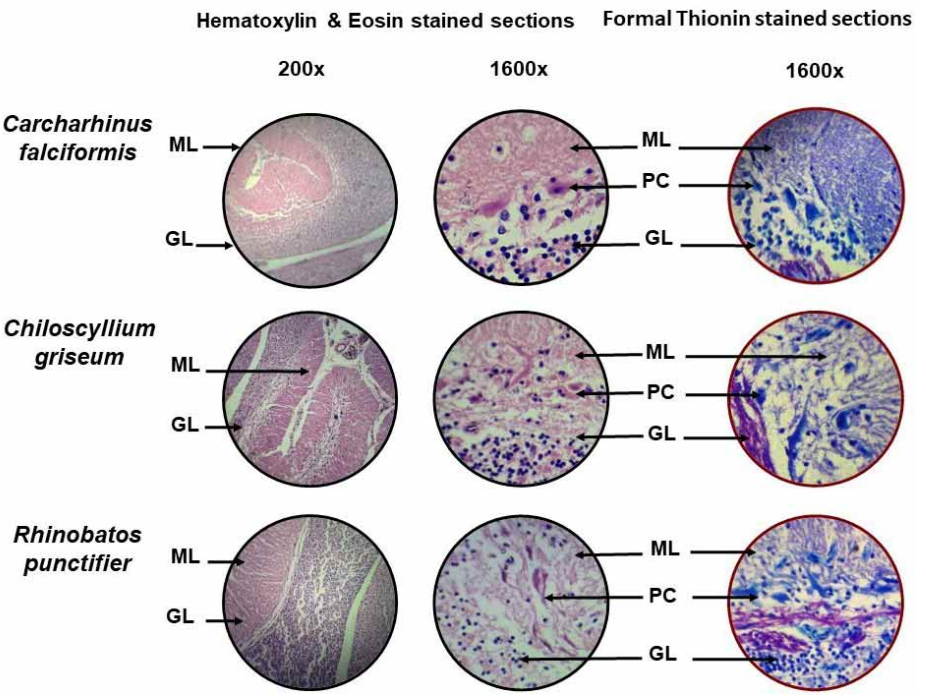

Fig. 2. Histomicrographs showing different layers of cerebellum of representative elasmobranch species in H&E-stained sections at 200x and 1600x magnifications. Formal Thionin stained sections at 1600x are also shown. Where, Purkinje cell granular layer and molecular layer are represented by PC, GL and ML, respectively.

apical processes projecting into ML. The molecular layer is made up of mainly fibers and sparsely distributed cells including both neurons and neuroglia. The granular layer is highly cellular comprising both neurons and neuroglia. The formal-thionin stained sections showed neuronal population in the three layers, stained as dark blue (Fig. 2).

Amongst examined species, *C. falciformis* showed high Molecular Layer Cell Count (MLCC), Molecular Layer Neuronal Count (MLNC) and low value of Molecular Layer Thickness (MLT). However, the difference in the total cellular count was not significant between any of the assessed species (Fig. 3A). Nevertheless, total neuronal count of *C. falciformis* was significantly high compared to *R. punctifier* (p=0.0313) (Fig. 3B). Conversely, MLT was found significantly less in *C. falciformis* compared to *R. punctifier* and *C. griseum* (p= <0.0001) (Fig. 3C). This in total suggests increased cellular density in ML of *C. falciformis* compared to other examined elasmobranch species.

Similar to ML, Granular Layer Cell Count (GLCC) and Granular Layer Neuronal Count (GLNC) was significantly high (p= 0.0002 and 0.0075, respectively) in *C. falciformis* compared to *R. punctifier* (Fig. 3D, E). Conversely, Granular Layer Thickness (GLT) of *C. falciformis* is significantly low compared to *R. punctifier* (p= <0.0001). Interestingly, *C. griseum* has the least thickness of the granular layer of all the examined species (Fig. 3F). This in total points to the increment in cellular density of sharks compared to rays.

The high Purkinje Cell Count (PCC) and low Interpurkinje distance (IPD) values were found in both *C. falciformis* and *C. griseum*, with no significant difference between the two shark species (Fig. 3G, H). In contrast to this, IPD is noticeably high in *R. punctifier* compared to *C. griseum* (p=0.0015) and *C. falciformis* (p= <0.0001). Relatively less PCC and more IPD in *R. punctifier* (ray) compared to sharks (*C. griseum* and *C. falciformis*) demonstrates increase in cellular density in sharks compared to rays. Nevertheless, diameter of PC is comparable between *R. punctifier* and *C. falciformis*, both being significantly less compared to *C. griseum* (Fig. 3I).

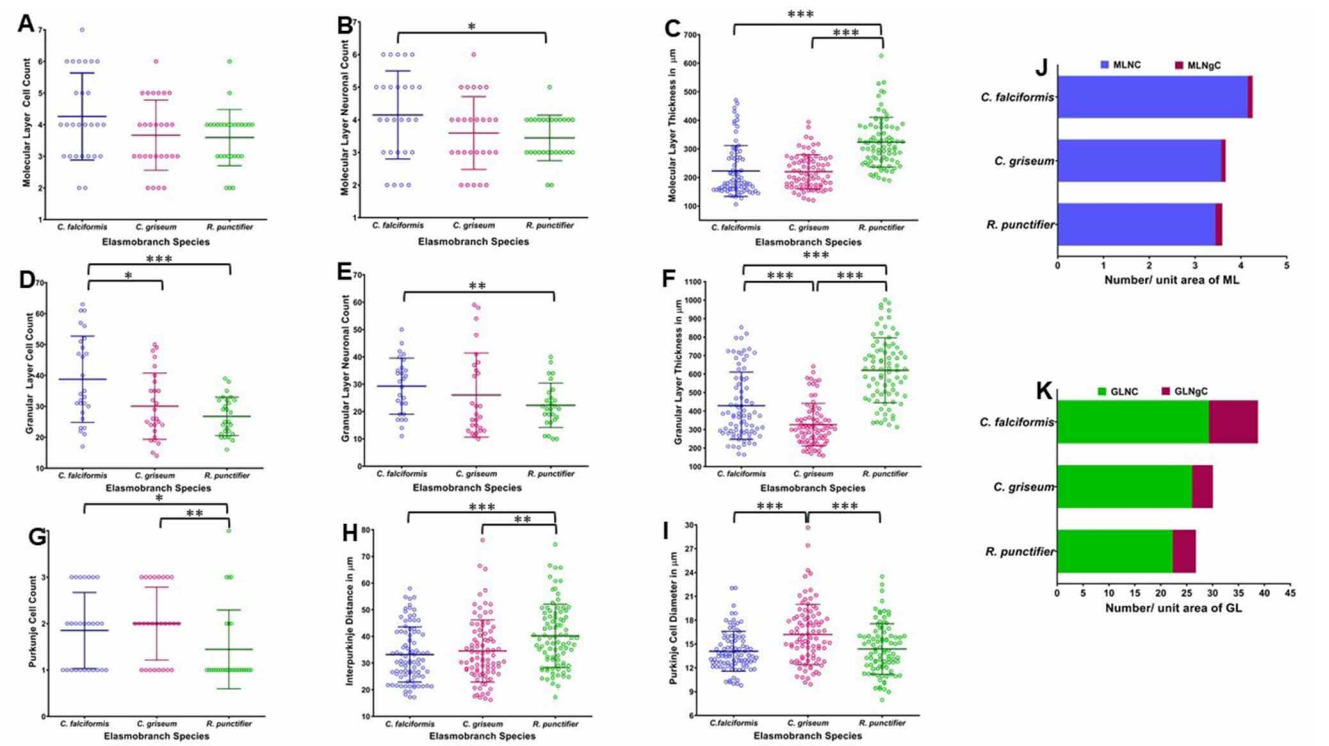

Fig. 3. Morphometry of different layers of elasmobranchs cerebellum. Graph showing differences in the (A) cellular count, (B) neuronal count and (C) thickness of molecular layer in the examined species of elasmobranchs. Graph showing differences between (D) cellular count, (E) neuronal count and (F) thickness of cerebellar granular layer in the examined elasmobranchs species. Graph showing differences between (G) cellular count, (H) interpurkinje distance and (I) cellular diameter of Purkinje neurons of the examined elasmobranchs species. Where data points mean and standard deviation are represented by circle, large and small horizontal lines, respectively. Statistical significances, where present, are represented by '*'. (J) Stacked bar graph represents proportion of neuron (blue) and neuroglia (brown) count in the molecular layer. (K) Stacked bar graph represents proportion of neuron (green) and neuroglia (brown) count in the granular layer of the examined elasmobranchs species.

Molecular Layer Neuroglia Count (MLNgC) was deduced by subtracting the MLNC from MLCC. The ratio of MLNgC to MLNC was found to be 1:23 in *R. punctifier* and 1:37 in both *C. griseum* and *C. falciformis* (Fig. 3J), suggesting comparable Neuroglia: Neuron proportion in sharks with noticeable difference from rays. Granular Layer Neuroglia Count (GLNgC) was derived by calculating the difference between GLCC and GLNC. The ratio of GLNgC and GLNC was 1:4, 1:4, and 1:9 in *R. punctifier*, *C. griseum* and *C. falciformis*, respectively (Fig. 3K). The ratio between *R. punctifier* and *C. griseum* was found comparable, however in *C. falciformis* GLNC was nearly twice compared to *C. griseum* and *R. punctifier*. Moreover, the ratio between neuroglia and neuron in both molecular layer and granular layer showed relatively more neuroglia support in granular layer, compared to molecular layer.

DISCUSSION

Vertical oscillations and residency of fishes within marine environment is driven by behavioral thermoregulation, hunting, navigation and swimming competency (Lisney & Collin, 2006). This enables fishes including member of elasmobranchs to be classified as benthic and pelagic. Given the differences in the niche, the requirement for the movement and by the same token coordination in both benthic and pelagic fishes are also noticeably different. Generally, the reef-associated species have a larger relative volume of telencephalon and cerebellum, whereas the deep-water species have a bigger volume of sensory centers like olfactory bulbs. (Yopak *et al*., 2010). Consistently, pelagic species of both sharks and teleosts, possess a relatively smaller cerebrum compared to coastal or reef-associated fishes (Lisney & Collin, 2006). In our study, decrease in NCV has been observed in the pelagic species (*C. falciformis*), compared to benthic species (*C. griseum*, *R. punctifier*) of same taxonomic group. However, the difference is not statistically significant. Thus, further analysis of more representative species may provide more resolved insights to the potential differences in cerebellar volume between benthic and pelagic species.

In *C. falciformis*, high cellular and neuronal count in cerebellar molecular and granular layers points to the evolution of highly developed "information processing neurons" which are stationed at GL and ML, providing connection to cerebellar cortex input and output neurons (New, 2001; Yopak *et al*., 2020). The GL is the input layer of the cerebellar cortex which receives information through mossy fibers (Laurens *et al*., 2013). Cerebellar Granule Cells are regarded as the most numerous neurons of brain (Yopak *et al*., 2020). They activate a microcircuit to target molecular layer interneurons (stellate cells in elasmobranchs), which play a vital role in motor behavior by controlling PC, the only output neurons of the cerebellar cortex (Dorgans *et al*., 2019). The observation of low MLT and GLT with higher MLCC and GLCC in *C. falciformis* suggests dense cellular packaging consistent with much resolved motor coordinating signals reflected by the navigatory and predatory nature of the specie (Galvan-Magana *et al*., 2019; Keller *et al*., 2021). Similarly, low cellular and neuronal counts with maximum MLT and GLT (less cellular packaging) in *R. punctifier* corresponds to its relatively benthic habitat and lifestyle (Harris *et al*., 1988).

Molecular layer neuroglia/neuron ratio was comparable between two species of *C. falciformis* and *C. griseum* but varies in *R. punctifier*. Similarly, Granular layer neuroglia/neuron ratio also suggested a high neuron number/unit area in *C. falciformis* (pelagic) compared to observed benthic species. Since the neuroglia by their function of myelination and synapse modulation influences the interneurons located in GL (Freeman & Doherty, 2006), this observation may be aligned with the lifestyle and high predatory nature of *C. falciformis* species.

Increased PCC with low IPD corresponds to low diameter of Purkinje cells (PCD) in *C. falciformis* and vice versa in *R. punctifier*. The observations suggest that count and diameter may counter balance in order to conserve the net PC inhibitory output signals. The finding is consistent to the earlier studies which reported no significant changes in the PCC, PCD and IPD over the course of 600MY of evolution (Herculano-Houzel, 2010). However high PCC, PCD and low IPD in *C. griseum* is not consistent with rest of the findings although it suggests increased cortical output signals in *C. griseum* specie of demersal benthic fishes. Therefore, analysis of more representative species may further resolve and provide insights to the neuronal architecture of cerebellar layers.

CONCLUSION

Histological analysis of three layers in examined species suggests that differences observed in the coordinated motor responses related to their habitat, navigation and prey capture can be linked to the differences found in granular layer organization or information processing unit. Moreover, high neuronal count in *C. falciformis* is consistent with voracious and navigator nature of sharks.

KHAN, N.; PERVEEN, K.; HUSSAIN, M.; QADEER, M. R. & SHARAFAT, S. Análisis histológico comparativo del cerebelo de especies representativas de elasmobranchii. *Int. J. Morphol., 41(2)*:383-388, 2023.

RESUMEN: En los peces elasmobranquios, las variaciones en la organización estructural general del cerebelo se han explorado ampliamente. Las características histológicas básicas del cerebelo, aunque se conservan en el grupo, pero la descripción comparativa de las variaciones celulares sutiles es limitada. El presente estudio tiene como objetivo explorar las variaciones histológicas y celulares en diferentes capas de la corteza cerebelosa de las especies representativas de elasmobranquios pertenecientes a diferentes hábitats. Nuestros hallazgos mostraron que la arquitectura histológica de la capa granular del cerebelo entre las especies examinadas varía notablemente. Se observó un gran aumento de la densidad celular en todas las capas del cerebelo en las especies representativas de tiburón en comparación con la raya. Luego, los hallazgos se compararon y discutieron con referencia a su hábitat y comportamiento.

PALABRAS CLAVE: Elasmobranquio; Cerebelo; Evolución; Carcharhinus; Chiloscyllium; Rinobatos.

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