

Extant cartilaginous fishes share trabecular and areolar mineralization patterns, but not tesserae, and evidence for a paedomorphic chimaera skeleton

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Abstract

A comprehensive study is lacking that clearly defines and directly compares the diverse mineralized endoskeletal tissues exhibited by extant chondrichthyans (elasmobranchs, such as sharks and skates, and holocephalans, such as chimaeras). Tiles of mineralized polygonal structures called tesserae occur at cartilage surfaces in chondrichthyans, but recent studies showing trabecular structures suggest that tesserae are not as common as previously thought. A specific region of tesserae termed cap zone and continuous (not tiled) mineralized elasmobranch neural arches demonstrate bone-like tissues. Areolar mineralized tissue in elasmobranchs is generally considered a unique chondrichthyan feature. Despite these reports, it remains unclear what mineralized endoskeletal features define extant chondrichthyans. To address this question, adult skeletal tissues in two elasmobranchs (little skate and small-spotted catshark) and a chimaera (spotted ratfish) were characterized using synchrotron radiation and desktop micro-CT imaging, and histological and immunofluorescent assays. Data from these extant chondrichthyan representatives suggested that trabecular and areolar mineralization, but not tesserae and bone-like tissues, are shared features of the extant chondrichthyan endoskeleton. Interestingly, three separate analyses argued that the chimaera endoskeleton retains ancestral embryonic features (i.e., paedomorphic). This study further proposes general terminology for character states of the extant chondrichthyan endoskeleton and infers those states in ancestral chondrichthyans.

eLife assessment

This study presents **valuable** research comparing three different species of extant cartilaginous fishes and describes new data on ratfish. The methods are **convincing** although the reviewers noted that standardized methods are essential when comparing numerical datasets. This study would be of interest to skeletal biologists working on the evolution of chondrichthyan skeletons.

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Introduction

The skeleton of chondrichthyans, or cartilaginous fishes, has long fascinated evolutionary biologists (Janvier, 1996 [↗](#); Maisey, 2013 [↗](#); Ørvig, 1951 [↗](#); Smith and Hall, 1990 [↗](#)). However, only in the past 15 years has an active research community revealed the histological and molecular complexity of extant chondrichthyan skeletal tissues. Even so, these studies are limited in scope, focussing mostly on specific skeletal features of the Elasmobranchii subclass of extant Chondrichthyes, such as sharks, skates, and rays (Atake et al., 2019 [↗](#); Dean et al., 2017 [↗](#); Eames et al., 2007 [↗](#); Johanson et al., 2012 [↗](#); Kemp and Westrin, 1979 [↗](#); Omelon et al., 2014 [↗](#); Porter et al., 2006 [↗](#); Seidel et al., 2016 [↗](#)), with only a couple including the Holocephali subclass, such as chimaera (Gillis et al., 2011 [↗](#); Herbert et al., 2022 [↗](#); Pears et al., 2020 [↗](#); Seidel et al., 2020 [↗](#); Smith et al., 2020 [↗](#)). Unfortunately, an in-depth description of the extant holocephalan endoskeleton is severely lacking, leading to a major deficit in understanding skeletal tissue character states across extant chondrichthyans.

Like most vertebrates, skeletal mineralization in extant chondrichthyans occurs by incorporation of biominerals into extracellular compartments of skeletal tissues (Golub, 2009 [↗](#); Lowenstam, 1981 [↗](#); Omelon et al., 2014 [↗](#)), giving rise to diverse mineralized tissue types in several anatomical sites of the chondrichthyan endoskeleton. Traditionally recognized as a defining endoskeletal feature of Chondrichthyes, tesserae form a mineralization pattern consisting of an array of distinct, polygonal units at the surface of cartilages (Kemp and Westrin, 1979 [↗](#); Maisey, 2013 [↗](#); Maisey et al., 2021 [↗](#)). In skates, however, a recently characterized trabecular mineralization pattern lies directly underneath the polygonal pattern of tesserae (Atake et al., 2019 [↗](#)). In fact, the cartilage surface in several endoskeletal regions of skates and rays only contain the trabecular mineralization pattern (Atake et al., 2019 [↗](#); Jayasankar et al., 2020 [↗](#)), suggesting that polygonal tesserae may not be as common as traditionally thought. Like polygonal tesserae, the little skate trabecular mineralization also forms an arrayed pattern, but the repeating units are in the form of trabecular struts interspersed by unmineralized regions, leading us to call them trabecular tesserae (Atake et al., 2019 [↗](#); Atake and Eames, 2021 [↗](#)). Descriptions of a trabecular mineralization pattern in the endoskeleton of sharks or chimaeras are currently lacking, leaving open the possibility that this is a phenotype specific to the Batoidea superorder (including skates and rays) of the subclass Elasmobranchii. In some chimaeras, the surfaces of cartilage in the anterior fused vertebrae (synarcual) and chondrocranium have sheets of mineralization that abut one another (Pears et al., 2020 [↗](#); Seidel et al., 2020 [↗](#)), but these do not have an arrayed pattern of repeating units, so perhaps they should not be called tesserae.

Recent evidence suggests that histological features of tesserae may vary according to the mineralization pattern. Traditional polygonal tesserae have distinct histological zones, with a cap zone superficial to a body zone near the surface of chondrichthyan cartilage (Atake et al., 2019 [↗](#); Berio et al., 2021 [↗](#); Kemp and Westrin, 1979 [↗](#); Seidel et al., 2016 [↗](#)). While skates have polygonal tesserae, trabecular tesserae in little skate have very small or even absent cap zones overlying extensive body zones (Atake et al., 2019 [↗](#)). Additionally, specific regions of tesserae termed spokes are hypermineralized and often devoid of cells, and spokes appear to be a common feature of tesserae regardless of their mineralization pattern (Atake et al., 2019 [↗](#); Pears et al., 2020 [↗](#); Seidel et al., 2020 [↗](#); Seidel et al., 2016 [↗](#)). Overall, these data strongly suggest that histological zones of tesserae actually correlate with mineralization patterns.

Some chondrichthyans have mineralized endoskeletal tissues that have been characterized as bone-like (Atake and Eames, 2021 [↗](#)). For example, the cap zone of tesserae displays bony features, like elongate cell lacunae in a mineralized ECM of densely packed Col1 fibers (Atake et al., 2019 [↗](#); Kemp and Westrin, 1979 [↗](#); Seidel et al., 2017 [↗](#); Seidel et al., 2016 [↗](#)). Unlike the cap zone, the body zone displays typical features of hyaline cartilage, which is the main cartilage type in

vertebrates (Goldring et al., 2006). The body zone consists of round chondrocyte lacunae embedded in a mineralized matrix abundant in loosely packed type II collagen (Col2) fibers and sulfated glycosaminoglycans (Enault et al., 2015; Seidel et al., 2017). As another example of bone-like tissue, many elasmobranch species have a non-tiled, mineralized perichondral tissue in their neural arches (Atake et al., 2019; Berio et al., 2021; Bordat, 1987; Eames et al., 2007; Kemp and Westrin, 1979; Seidel et al., 2016). The neural arch bone-like tissue demonstrates morphological and histological features of perichondral bone, including a compact mineralization pattern, elongate cell lacunae, canaliculi-like channels connecting adjacent lacunae, and tightly packed type I collagen (Col1) fibers (Bordat, 1987; Eames et al., 2007; Kemp and Westrin, 1979; Peignoux-Deville et al., 1982; Rossert and de Crombrughe, 2002; Seidel et al., 2017). Bone-like tissues might be a defining feature of extant chondrichthyans, but relevant data from chimaeras are needed.

Finally, areolar mineralized tissue is generally considered a unique feature of the extant chondrichthyan endoskeleton, characterized mostly in elasmobranchs as large sheets of mineralization in the notochordal sheath of the vertebral body (centrum) (Didier, 1995; Ridewood and MacBride, 1921). Areolar mineralized tissue typically contains concentric lamellae of elongate lacunae in a fibrocartilage-like extracellular matrix and a compact mineralization pattern (Atake et al., 2019; Criswell et al., 2017; Dean and Summers, 2006; Eames et al., 2007). Mineralized centra are not thought to be common in chimaeras, but when present, they can appear as multiple mineralized rings in each vertebral segment, which contrasts with the grossly biconcave morphology of segmented centra in elasmobranchs (Gadow and Abbott, 1895).

With hopes of revealing shared and clade-specific features among chondrichthyans, here we clarify many of the unresolved issues highlighted above. Testing the hypothesis that extant chondrichthyans share histological and morphological features of endoskeletal mineralized tissues, adult tissues in several endoskeletal regions of the small-spotted catshark *Scyliorhinus canicula* (shark), little skate *Leucoraja erinacea* (skate), and spotted ratfish *Hydrolagus coliei* (chimaera) were characterized using synchrotron radiation and desktop micro-CT imaging, and histological and immunofluorescent assays. The data argue that trabecular and areolar, but not polygonal, mineralization patterns are shared by extant chondrichthyans. For ratfish, tesseral mineralization, cap zones, and neural arch bone-like tissues were absent, and three separate analyses argued that this chimaera displays skeletal paedomorphosis, or retention of ancestral embryonic features (Garstang, 1922). While additional chondrichthyan representatives can bolster these findings, this study proposes general terminology for character states of the extant chondrichthyan endoskeleton, concluding that ancestral chondrichthyans had trabecular and areolar mineralization patterns, with tesserae likely lost in various extant chondrichthyan clades.

Results

Adult mineralized tissues in spotted ratfish had lower tissue mineral density (TMD) than little skate or small-spotted catshark

To assess whether chimaeras demonstrate skeletal mineralization in anatomical sites that are homologous to those of elasmobranchs, 3D rendering of micro-CT images of anterior regions of adult ratfish were qualitatively and quantitatively analyzed (Fig. 1A,B). Micro-CT images revealed mineralized tissues in several endoskeletal regions including segmented vertebral neural arches and centra (Fig. 1C,D), several skeletal elements in the pharyngeal skeleton (Fig. 1E), and the fused vertebral synarcual (Fig. 1F,G). Although elasmobranchs mineralize neural arches in their precaudal and caudal vertebrae (Atake et al., 2019; Berio et al., 2021; Eames et al., 2007), mineralized neural arches were present mainly in precaudal vertebrae of ratfish (Fig. 1C,D; data not shown). Mineralized skeletal elements in the pharyngeal skeleton included the

basihyal, Meckel's, ceratohyal, basibranchial, hypobranchials, and ceratobranchial (Fig. 1E). Spinal nerve foramina are common in the synarcual of cartilaginous fishes and were present in the ventral sides of the ratfish synarcual (Fig. 1F; Claeson, 2011; Johanson et al., 2015; Pears et al., 2020). Unlike the synarcual in skates and other batoids, where distinct vertebral parts are not discernible (Atake et al., 2019; Claeson, 2011), the synarcual in ratfish had distinct mineralized centra (Fig. 1G).

TMDs of the synarcual, neural arches, and centra in ratfish, little skate, and catshark were quantitated and compared, where possible, since sharks do not have a synarcual (Fig. 1H). To make TMD analysis of the synarcual in ratfish and little skate more comparable, distinct centra in the ratfish synarcual were segmented and excluded. The average TMD of the ratfish synarcual was 0.31 ± 0.11 gHA/cm³, significantly lower than the TMD of the little skate synarcual, which was 0.94 ± 0.04 gHA/cm³ (Fig. 1H; $p = 6.5 \times 10^{-4}$). Similarly, the TMD of ratfish neural arches (0.40 ± 0.05 gHA/cm³) was significantly lower than the TMD of neural arches of either little skate (0.96 ± 0.04 gHA/cm³; $p = 4.5 \times 10^{-7}$) or catshark (0.81 ± 0.04 gHA/cm³; $p = 9 \times 10^{-6}$) (Fig. 1H). The average TMD of ratfish centra (0.42 ± 0.10 gHA/cm³) was also significantly lower than the TMD of centra of either little skate (0.88 ± 0.05 gHA/cm³; $p = 3.4 \times 10^{-5}$) or catshark (0.84 ± 0.02 gHA/cm³; $p = 1.1 \times 10^{-4}$) (Fig. 1H). TMD of segmented centra within the ratfish synarcual was similar to that of centra in segmented vertebrae of ratfish (0.46 ± 0.11 gHA/cm³). These analyses showed that the synarcual, neural arches, and centra in ratfish had significantly lower TMDs compared to homologous elements in little skate and catshark.

Neural arches in ratfish lacked bone-like tissue

To clarify whether chimaeras have bone-like tissue like elasmobranchs, micro-CT and histological analyses of neural arches in segmented precaudal vertebrae of ratfish were compared to neural arches in segmented precaudal vertebrae of little skate and catshark. Unlike the compact mineralization pattern displayed by neural arch bone-like tissue in little skate and catshark (Fig. 2A,B; Atake et al., 2019; Berio et al., 2021), mineralization of neural arches in ratfish was organized in an irregular pattern of small compact and trabecular regions (Fig. 2C). Neural arch bone-like tissues in little skate and catshark were extensively mineralized and surrounded a cartilaginous core (Fig. 2D,E,G,H). By contrast, mineralized tissues in ratfish were restricted to the medial and lateral periphery of the cartilaginous neural arches (Fig. 2F,I).

Histologically, mineralization of continuous, bone-like tissues in neural arches of little skate and catshark was marked by Alizarin red (Fig. 2G,H). Alizarin red staining also marked mineralized tissues in ratfish neural arches, but they were much thinner and not continuous (Fig. 2I). Stained with both Alizarin red and Alcian blue, the neural arch core in little skate was mineralized cartilage, while that of the catshark was mostly unmineralized cartilage (Fig. 2G,H). Alcian blue staining also showed that most of the neural arches in ratfish was unmineralized cartilage (Fig. 2I). Birefringence of tissue fibers was enhanced using picrosirius red staining and observed under polarized light (Eames et al., 2007). Birefringent fibers (likely reticular) were observed in muscles lateral to neural arches in all three species (Fig. 2J-L). Birefringence of tightly packed collagen fibers in neural arch bone-like tissues was observed in little skate and catshark (Fig. 2J,K), but no birefringence was observed in ratfish neural arches (Fig. 2L). Only the cartilaginous core in neural arches of little skate and catshark demonstrated Col2 immunofluorescence (Fig. 2J,K), whereas most regions (including unmineralized cartilage and mineralized tissue) in ratfish neural arches demonstrated Col2 immunofluorescence (Fig. 2L). These results showed that bone-like tissue was absent in neural arches of ratfish, and the irregular pattern of mineralized tissue in ratfish neural arches was mineralized cartilage.

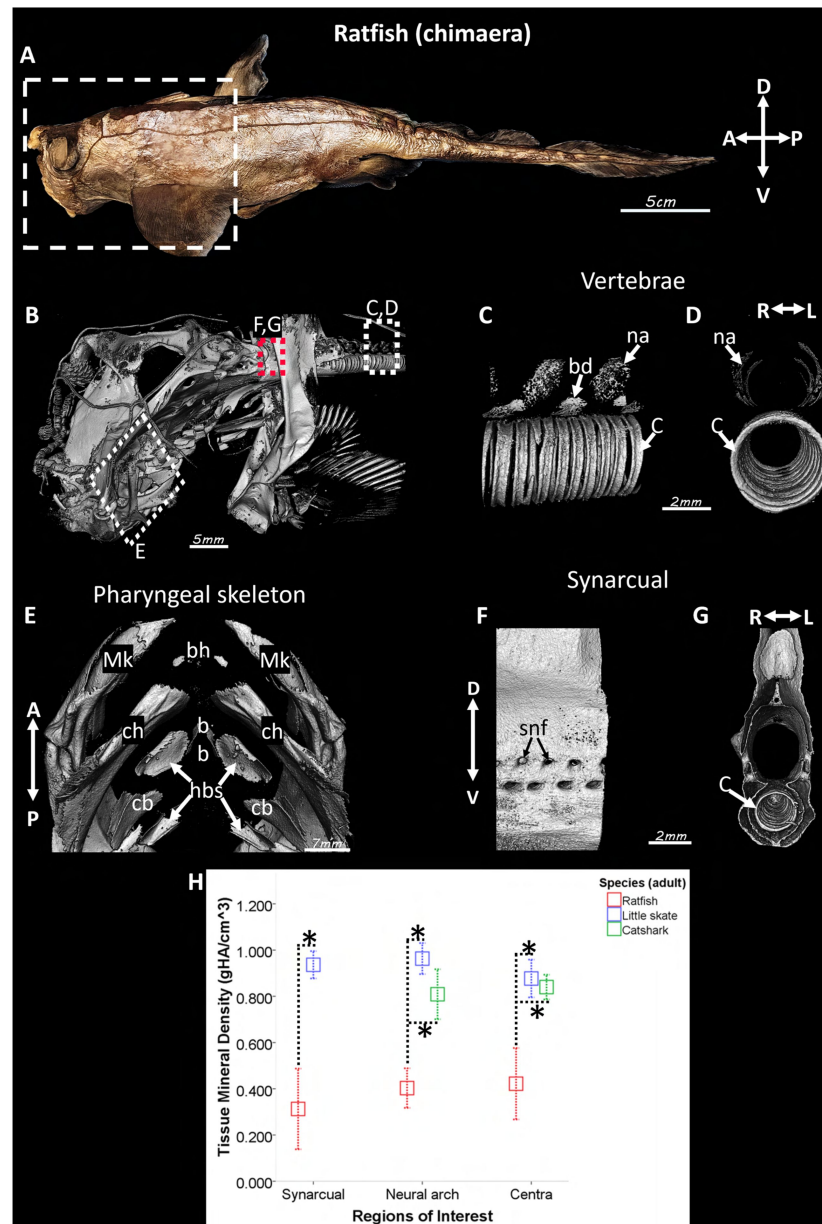


Figure 1.

Mineralized tissues were abundant in the spotted ratfish, but they had lower tissue mineral density (TMD) compared to the little skate and the small-spotted catshark. **(A)** Photograph of the ratfish *Hydrolagus colliei* illustrating the anterior region that was micro-CT imaged. **(B–G)** Micro-CT 3D renderings of the anterior region of ratfish **(B)** showed mineralized tissues in neural arches, basidorsals, and centra in segmented vertebrae **(C,D)**, in the ceratohyal and other skeletal elements in the pharyngeal skeleton **(E)**, and in the fused vertebral synarcual **(F,G)**. **(H)** Comparative quantitative analyses showed that TMD of the synarcual, neural arch, and centra in ratfish were significantly lower than TMDs of same regions in little skate and catshark. Abbreviations: A, anterior; bb, basibranchial; bd, basidorsal; bh, basihyal; c, centrum; cb, ceratobranchial; ch, ceratohyal; D, dorsal; hbs, hypobranchials; L, left; MK, Meckel's; na, neural arch; P, posterior; R, right; snf, spinal nerve foramina; V, ventral. * indicates statistically significant comparison ($p < 0.05$). Scale bars: as indicated.

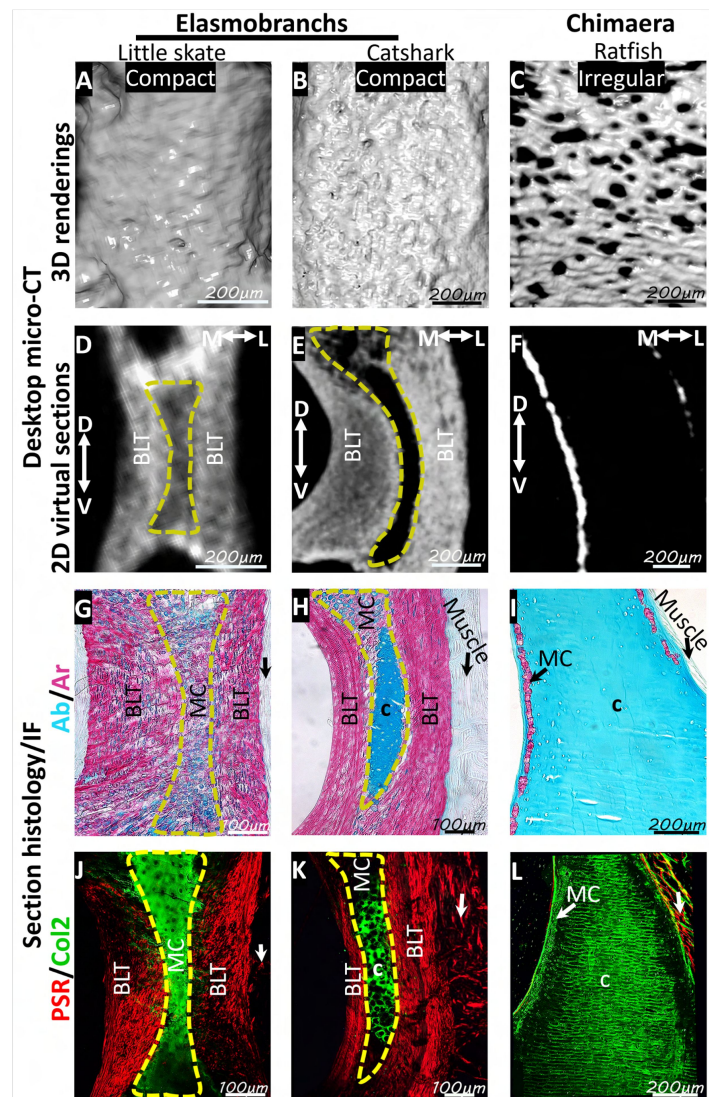


Figure 2.

Ratfish lack neural arch bone-like tissue. (A-C) Micro-CT 3D renderings showed a compact mineralization pattern of bone-like tissue in neural arches of segmented vertebrae in little skate (A) and catshark (B), and an irregular pattern of mineralized tissues in ratfish (C). (D-F) Micro-CT 2D virtual slices showing that the bone-like tissue in little skate and catshark was extensively mineralized and surrounded a neural arch core (D,E), whereas irregular mineralized tissues in ratfish neural arches were restricted to medial and lateral periphery (F). (G-L) Section histology showed Alizarin red staining of continuous mineralization and birefringent collagen fibers in neural arch bone-like tissues in little skate (G,J) and catshark (H,K), but not in ratfish (I,L). Picrosirius red staining also revealed some birefringent fibers (likely reticular) in muscles on the lateral sides of neural arches in little skate, catshark, and ratfish (J-L). Irregular mineralized tissue in the ratfish neural arch was discretely organized, Alizarin red-positive, and Col2-positive mineralized cartilage (I,L). Other regions of ratfish were Alcian blue-positive and Col2-positive unmineralized cartilage (I,L). The neural arch core in little skate and catshark consisted of mineralized cartilage (MC) and mostly unmineralized cartilage, respectively (J,K). Abbreviations: 2D, two-dimensional; 3D, three-dimensional; Ab, Alcian blue; Ar, Alizarin red; BLT, bone-like tissue; c, cartilage; Col2, collagen type 2; D, dorsal; IF, immunofluorescence; L, lateral; M, medial; MC, mineralized cartilage; PSR, picrosirius red; V, ventral. Scale bars: as indicated.

Trabecular mineralization was present in little skate, catshark, and ratfish

To clarify whether polygonal or trabecular tesserae are defining endoskeletal features of extant chondrichthyans, morphological patterns and histological features of mineralized tissues were characterized in several endoskeletal regions, including hyomandibulae and precaudal vertebrae of little skate, caudal vertebrae of catshark, and ceratohyals of ratfish (**Fig. 3A-D**). Similar to many other skeletal elements in elasmobranchs (Atake et al., 2019; Kemp and Westrin, 1979; Maisey, 2013; Marrama et al., 2019; Seidel et al., 2016; Wilga and Ferry, 2015), desktop micro-CT renderings of hyomandibulae in little skate showed polygonal tesserae, displaying a superficial, arrayed pattern of repeating polygonal units (**Fig. 3A,E**; Atake et al., 2019; Kemp and Westrin, 1979; Maisey, 2013; Marrama et al., 2019; Seidel et al., 2016; Wilga and Ferry, 2015). In little skate, an arrayed pattern of repeating trabecular units was located underneath the superficial polygonal tesseral pattern (**Fig. 3E**; Atake et al., 2019). In multiple regions examined in catshark (vertebrae and ceratohyals) and ratfish (vertebrae, pharyngeal skeleton, and the synarcual), a polygonal mineralization pattern was not observed. Non-tesseral mineralization patterns were observed in some regions of the catshark and ratfish. For example, mineralized tissues in catshark haemal arches and ratfish ceratohyals had a trabecular pattern, like that of trabecular tesserae in the vertebral neural spine of little skate, but the trabecular pattern in catshark and ratfish was not as clearly organized into arrays of repeating trabecular units (**Fig. 3F-H**).

To assess whether trabecular mineralization in catshark and ratfish was histologically similar to trabecular mineralization of tesserae in little skate, features of histological cross sections were compared to tesserae in little skate. Like polygonal and trabecular tesserae in little skate, Alcian blue and Alizarin red staining showed mineralized tissues in discrete perichondral regions of catshark haemal arches and ratfish ceratohyals (**Fig. 3I-L**; perichondrium marked by straight dashed line). In little skate, a bone-like cap zone was just below the perichondrium, stained more intensely than the body zone with Alizarin red, and was present in both polygonal and trabecular tesserae (**Fig. 3I,J**). In catshark and ratfish, bone-like cap zones were absent, and an unmineralized, Alcian blue-positive cartilage separated Alizarin red-positive mineralized tissues from overlying perichondrium (**Fig. 3K,L**). Matrix of the little skate bone-like cap zone was fast green-positive and had birefringent collagen fibers, but these staining patterns were not observed in catshark and ratfish (**Fig. 3M-T**). Instead, regions just below the perichondrium of catshark haemal arches and ratfish ceratohyals were Safranin O-positive and Col2-positive cartilage (**Fig. 3O,P,S,T**). Staining patterns near the cartilage surface of catshark and ratfish were consistent with those of mineralized cartilage in the tesseral body zone of the little skate, although chondrocyte lacunae in these regions were not obvious in ratfish (**Fig. 3I-T**). Therefore, histological features of trabecular mineralized tissues in catshark haemal arches and ratfish ceratohyals were similar to only the body zones of tesserae in the little skate, which consisted of mineralized cartilage.

Cap zones correlated with polygonal tesseral mineralization patterns, while body zones correlated with trabecular mineralization patterns

Given that trabecular tesserae typically have small or even absent cap zones, and polygonal tesserae typically have large cap zones, histology and micro-CT segmentation were used to see whether tesseral histological features correlated with tesseral mineralization patterns. In little skate, the larger and smaller cap zones in polygonal and trabecular tesserae, respectively, were similarly marked by Trichrome's acid fuchsin and elongated lacunae (**Fig. 4A,F**). Spokes were located below the cap zone, distinguished on histological sections by the facts that they often

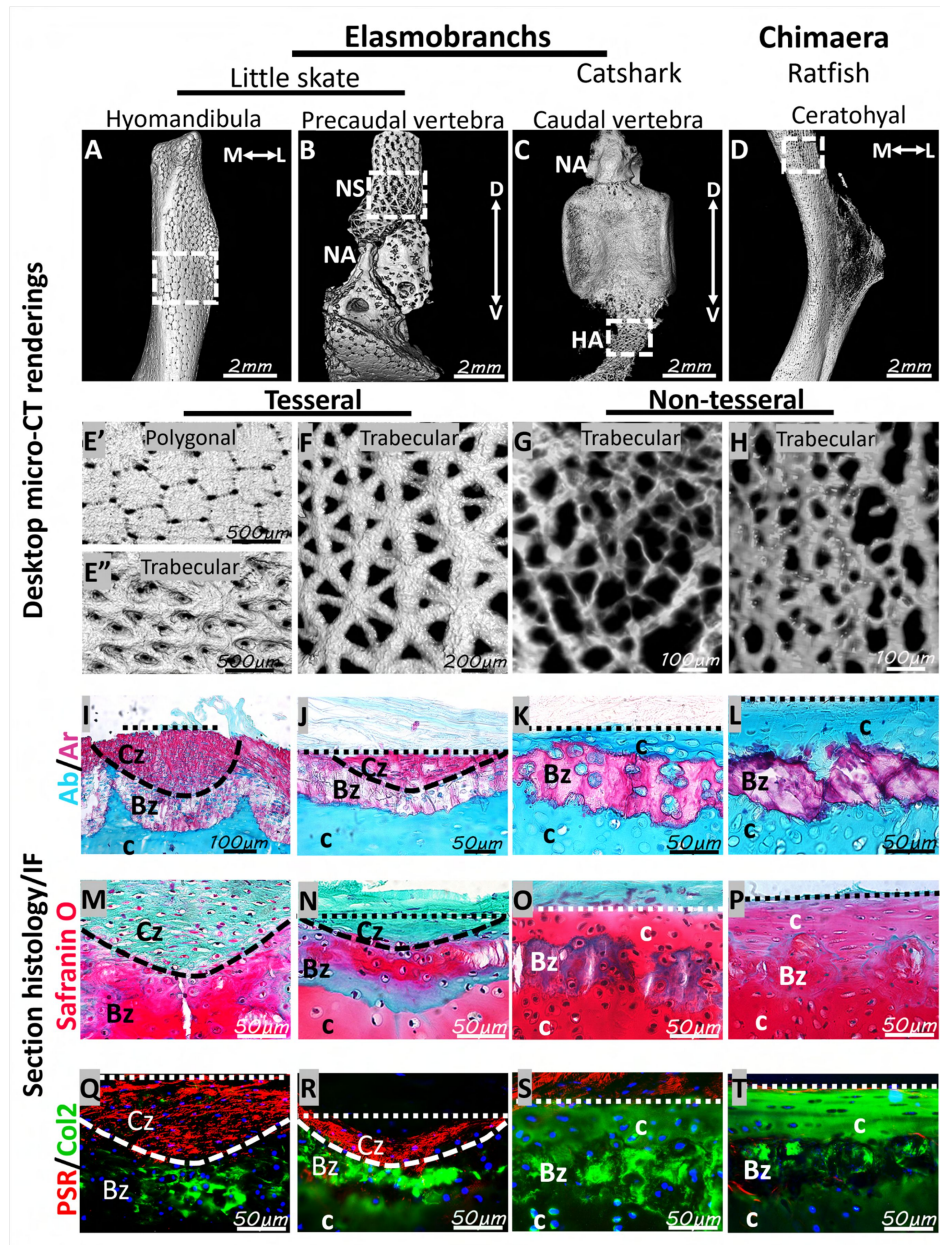


Figure 3.

Little skate, catshark, and ratfish all showed trabecular mineralization but without the bone-like cap zone in catshark and ratfish. (A-D) Micro-CT renderings of the little skate hyomandibula and precaudal vertebra (A,B), catshark caudal vertebra (C), and ratfish ceratohyal (D). (E-H) Tesserall and non-tesserall mineralization patterns in little skate, catshark, and ratfish. The little skate hyomandibula displayed a superficial polygonal tesserall pattern (E') and an underlying trabecular tesserall pattern (E''), while regions of the little skate neural spine only displayed a trabecular tesserall pattern (F). The catshark haemal arch (G), and ratfish ceratohyal (H) displayed a non-tesserall trabecular pattern. Regions for hi-mag views in panels E'-H are indicated in corresponding panels of A-D. (I-T) Histological and immunofluorescent features of tesserall or non-tesserall mineralized tissues in little skate (I,J,M,N,Q,R), catshark (K,O,S), and ratfish (L,P,T). Alizarin red staining, Safranin O staining, and Col2 immunofluorescence showed that little skate, catshark, and ratfish had the body zone of mineralized cartilage (I-T). The bone-like tesserall cap zone consisting of elongate lacunae in matrix that is mineralized (I,J), Safranin O-negative (M,N), Col2-negative (Q,R), and contains birefringent collagen fibers (Q,R) was present only in polygonal tesserall and trabecular tesserall in little skate. Abbreviations: Ab, Alcian blue; Ar, Alizarin red; Bz, body zone; c, cartilage; Col2, collagen type 2; Cz, cap zone; D, dorsal; IF, immunofluorescence; L, lateral; M, medial; PSR, picrosirius red; NA, neural arch; NS, neural spine; V, ventral. Scale bars: as indicated.

appeared as tears or sectioning artifacts when demineralized, and they did not stain strongly with any Trichrome dye (**Fig. 4A,F**). Non-spoke regions of the body zone stained either slightly blue with Trichrome's aniline blue or magenta with Trichrome's acid fuchsin.

Enough histological features of tesserae were also visible in synchrotron radiation micro-CT renderings, making it possible to segment and analyze the spatial correlation between tesseral mineralization patterns and histological features (**Fig. 4A-J**). Segmentation of 3D renderings of polygonal tesserae showed clearly that the cap zones were responsible for the superficial polygonal mineralization pattern (red in **Fig. 4B-D**). Hypermineralized spokes were restricted to the body zone, and their segmentation from 3D renderings demonstrated that they were responsible for the deeper trabecular mineralization pattern of polygonal tesserae (blue in **Fig. 4B,C,E**). Unlike polygonal tesserae, trabecular tesserae in little skate displayed identical trabecular mineralization patterns whether viewed from the superficial or deep surfaces of the cartilage (**Fig. 4I,J**). The smaller cap zones in trabecular tesserae were discernible on the superficial surface, but they were not as laterally extensive as cap zones of polygonal tesserae (**Fig. 4C,D,H,I**). Thus, larger and laterally extensive cap zones appeared to be the reason why polygonal tesserae displayed a superficial polygonal mineralization pattern. Trabecular mineralization patterns in both polygonal and trabecular tesserae appeared to derive mostly from spokes, but also from non-spoke regions, within body zones.

A cap zone was absent in ratfish, but Trichrome staining patterns and other histological features of the body zone and spokes were similar to tesserae in little skate, so correlations between spokes and trabecular mineralization patterns were also analyzed (**Fig. 4K-O**). Some histological features of tesserae, such as body zones, spokes, and intertesseral fibers, were apparent in the trabecular mineralization pattern of the ratfish ceratohyal (**Fig. 2H**; **Fig. 4K**; Seidel et al., 2020). Like trabecular tesserae in little skate, the superficial and deep surfaces of the trabecular mineralization in ratfish were identical (**Fig. 4I,J,N,O**). However, segmentation of 3D renderings showed that spokes in ratfish were patchy and did not display the arrayed pattern seen in trabecular tesserae of little skate (**Fig. 4A-O**). Therefore, compared to little skate, the less-organized spokes in body zones of ratfish correlated with the non-tesseral trabecular mineralization pattern.

Ratfish centra contained areolar mineralized tissue

Some chimaeras were reported to mineralize their centra like elasmobranchs, but areolar mineralized tissue has never been carefully characterized in chimaeras (Didier, 1995; Gadown and Abbott, 1895; Ridewood and MacBride, 1921). To clarify whether chimaeras have areolar mineralized tissue, morphological and histological features of ratfish centra were compared to those of little skate and catshark, two elasmobranch species who are recognized to have areolar mineralization (Atake et al., 2019; Clement, 1992; Criswell et al., 2017). Like elasmobranchs, each centrum in ratfish precaudal vertebrae demonstrated a compact mineralization pattern (**Fig. 5A-C**). However, precaudal vertebrae in ratfish had multiple rings of centra per vertebral segment (i.e., unit with paired neural arches and basidorsals), and no ratfish centrum displayed the typical biconcave gross morphology associated with elasmobranch centra (**Fig. 5A-C**).

Alizarin red section histology showed that centra in little skate, catshark, and ratfish comprised of rings of mineralized tissue spanning different numbers of centrum layers (**Fig. 5D-I**). Areolar mineralized tissues in the middle layer of the centrum of little skate, catshark, and ratfish had elongate cell lacunae organized in concentric lamellae (inserts in **Fig. 5G-I**; Atake et al., 2019; Criswell et al., 2017; Eames et al., 2007), and they were birefringent (data not shown). In addition to areolar mineralized tissue in the middle layer of the centrum, inner and outer layers of the little skate centrum contained mineralized cartilage (**Fig. 5G,J,M**), while the outer layer of the catshark centrum had mineralized cartilage (**Fig. 5H,K,N**). Markers of cartilage differed in areolar mineralized tissue among little skate, catshark, and ratfish. In little skate, areolar mineralized tissue did not display Safranin O staining or Col2 immunofluorescence (**Fig. 5J,M**).

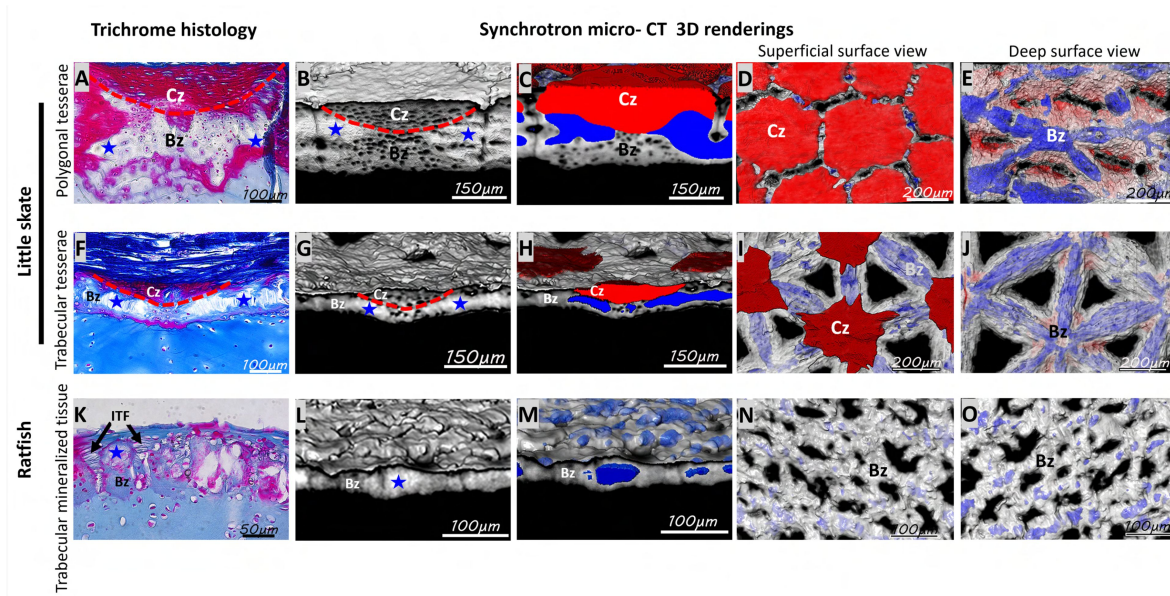


Figure 4.

Micro-CT segmentation clearly correlated histological features of mineralized tissues with their mineralization patterns. (A-J) Trichrome's acid fuchsin marked the larger and smaller cap zones of little skate polygonal and trabecular tesserae, respectively (A,F). 3D renderings of polygonal tesserae showed that the cap zone (Cz, segmented in red) was superficial and reflected the polygonal pattern (B-D). The smaller cap zone in trabecular tesserae was not laterally extensive enough to display a superficial polygonal pattern like that of polygonal tesserae (B-D,G-I). Spokes in polygonal and trabecular tesserae did not stain with Trichrome (* in A,F). 3D renderings showed that spokes (*, segmented in blue) in the deeper body zones displayed a radiating pattern like the trabecular patterns in little skate polygonal and trabecular tesserae (A-C,E,F-J). (K-O) Trichrome staining demonstrated that trabecular mineralized tissue in ratfish lacked a cap zone but had other features, such as a body zone and intertesseral fibres (K). Segmentation demonstrated that spokes in ratfish were patchy and did not display a radiating trabecular pattern (K-O). Abbreviations: Bz, body zone; Cz, cap zone; ITF, intertesseral fibres. Scale bars: as indicated.

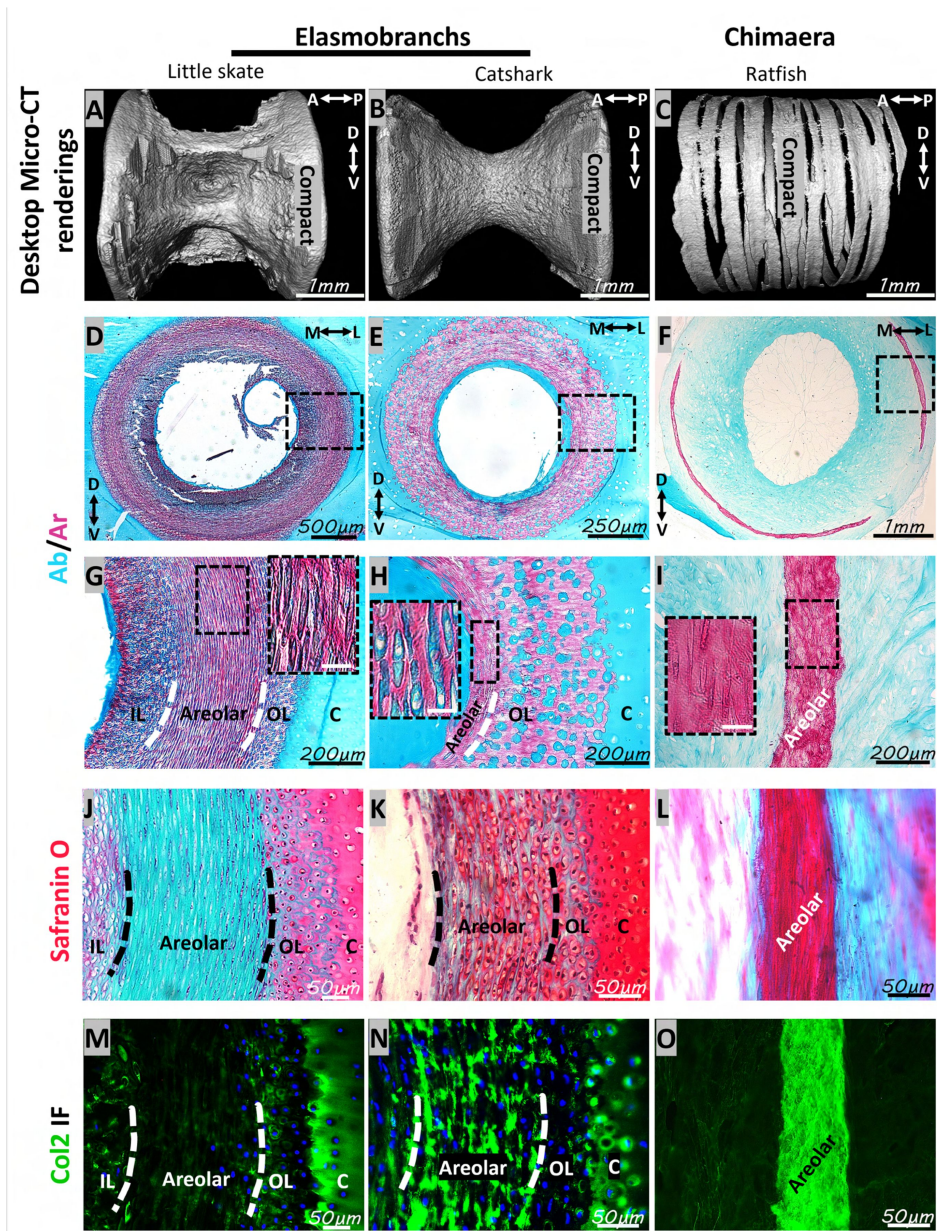


Figure 5.

Ratfish centra had the same features as areolar mineralized tissues in little skate and catshark. (A-C) Micro-CT renderings demonstrated that centra in little skate and catshark had a compact mineralization and a gross biconcave morphology (A,B), while centra in ratfish also displayed a compact mineralization pattern but were organized in multiple rings (C). (D-O) Consistent with areolar mineralized tissue in little skate (D,G) and catshark (E,H), Alcian blue/Alizarin section histology (Ab/Ar) showed that mineralized centra in ratfish consisted of a mineralized areolar centra layer with elongate lacunae in concentric lamellae (F,I). Regions for hi-mag views in panels G-O are indicated in corresponding panels of D-F; regions for hi-mag inserts in panels G-I are indicated in those panels. Areolar mineralized tissue in little skate lacked Safranin O staining and Col2 immunofluorescence (J,M), while catshark areolar mineralized tissue demonstrated weak Safranin O staining and Col2 immunofluorescence (K,N). Safranin O staining and Col2 immunofluorescence highlighted cartilaginous matrix of areolar mineralized tissue in ratfish (L,O). Little skate also had mineralization in both outer and inner centra layers (G), while catshark also had an outer mineralized layer (H), and these additional centra layers demonstrated weak Safranin O staining and Col2 immunofluorescence (J,M,K,N). Abbreviations: A, anterior; Ab, Alcian blue; Ar, Alizarin red; c, cartilage; Col2, collagen type 2; D, dorsal; IF, immunofluorescence; IL, inner layer; L, lateral; M, medial; OL, outer layer; P, posterior; V, ventral. Scale bars: as indicated; inserts in G-I are 20 µm.

In catshark, areolar mineralized tissue demonstrated weak Safranin O staining and Col2 immunofluorescence (**Fig. 5K,N**). By contrast, areolar mineralized tissue in ratfish had relatively strong Safranin O staining and Col2 immunofluorescence (**Fig. 5L,O**). Overall, these data suggested that ratfish have areolar mineralized tissue, which had been previously characterized mostly in elasmobranchs (Criswell et al., 2017; Eames et al., 2007; Ridewood and MacBride, 1921).

Embryological analyses of little skate centra suggested that adult ratfish had paedomorphic centra

To shed light on differences in morphology between adult elasmobranch and ratfish centra (**Fig. 5A-C**), morphological and histological features of centra in a developmental series of little skate were compared to those of adult ratfish. Centra in elasmobranchs are thought to acquire a biconcave morphology during development when the perichordal sheath becomes constricted by mesenchymal cells (Gadow and Abbott, 1895). In little skate embryos and juveniles, desktop micro-CT renderings showed mineralization in the centrum and pairs of neural arches, basidorsals, and haemal arches of each vertebral segment (**Fig. 6A-C**). Expanding upon recent work showing that anterior and posterior segments of somites fuse during skate vertebral development, similar to tetrapods (Criswell and Gillis, 2020), independent anterior and posterior mineralizations of each interneural appeared to later fuse during development. In ratfish, each vertebral segment of paired neural arches and basidorsals had an average of six centra (**Fig. 6D**). Synchrotron radiation (SR) micro-CT renderings showed that centra in little skate stage 32 embryos were slightly constricted in the middle of each element's anterior-posterior axis, and this constriction progressed through 6.5 cm disc width juveniles, when the classic biconcave morphology became obvious (**Fig. 6E-G**). SR micro-CT renderings showed that adult ratfish centra were constricted to a similar extent as centra in little skate embryos (**Fig. 6E,F,H**). In little skate embryos, Alcian blue and Alizarin red section histology showed that the areolar mineralized tissue initiated in the middle layer of the centrum and had elongate cell lacunae organized in concentric lamellae, similar to that observed in adult ratfish (**Fig. 5F**; **Fig. 6I,J**). At later developmental stages of little skate, such as 6.5 cm DW juveniles, the outer layer of the centrum also demonstrated mineralized cartilage (**Fig. 6K**). These morphological and histological similarities of centra in little skate embryos and adult ratfish suggested that adult ratfish retained an embryonic form of centra, a phenomenon termed paedomorphism (Garstang, 1922).

To further test the possibility that ratfish had paedomorphic centra, TMDs of centra in the developmental series of little skate were compared to that of adult ratfish, because TMD of skeletal elements tends to increase during development (**Fig. 1H**; Forbes, 1976). TMD of little skate centra increased significantly from stage 32 to stage 33 embryos ($p = 9.5 \times 10^{-3}$), while TMD of 6.5 cm DW juveniles was only significantly higher than TMD of stage 32 embryos (**Fig. 6L**; $p = 9.6 \times 10^{-4}$). TMD of adult ratfish centra was significantly higher than TMD of centra in little skate stage 32 embryos, but it was statistically indistinguishable from TMD of centra in little skate stage 33 embryos and 6.5 cm DW juveniles (**Fig. 6L**). In addition to morphological similarities, similar TMDs of centra from little skate embryos and adult ratfish argued strongly that ratfish had paedomorphic centra.

Discussion

Which tissues are common in the extant chondrichthyan endoskeleton? Bone-like and areolar tissues have been characterized in several members of the chondrichthyan subclass Elasmobranchii, such as sharks, skates, and rays, (Atake et al., 2019; Berio et al., 2021; Bordat, 1987; Kemp and Westrin, 1979; Ørvig, 1951; Peignoux-Deville et al., 1982; Seidel et al., 2016). In addition, polygonal tesserae are traditionally associated with chondrichthyans (Atake

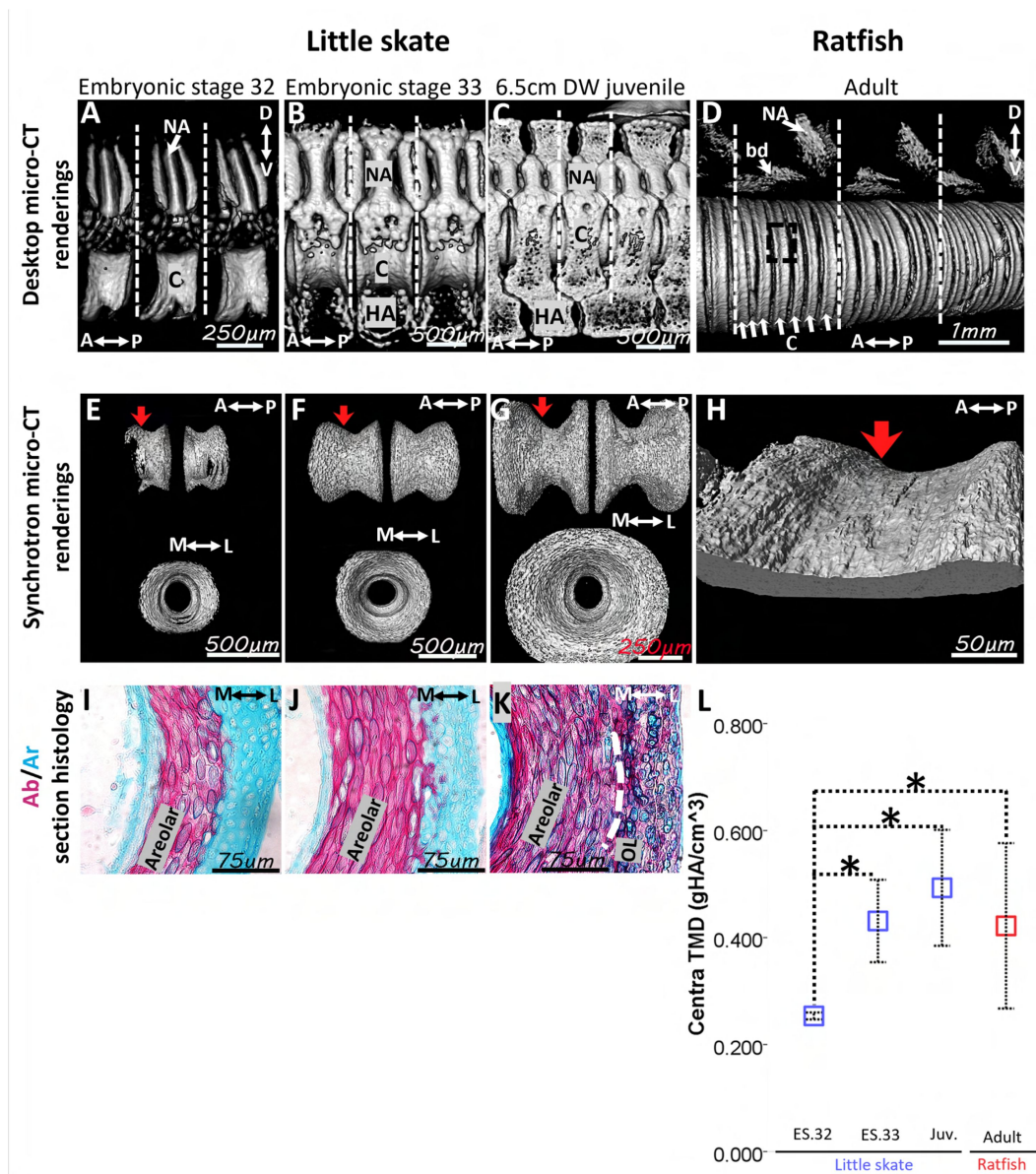


Figure 6.

Morphological and histological features of centra in little skate embryos and juveniles suggested that the adult ratfish demonstrated pedomorphic centra. (A–D) Desktop micro-CT renderings showed centrum and other mineralized structures in vertebral segments (dashed lines) of little skate embryos and juveniles (A–C) and adult ratfish (D). (E–H) Synchrotron radiation micro-CT renderings of centra in little skate embryos and juveniles (E–G) and adult ratfish (H) showed that centra in the ratfish were slightly constricted like those of little skate embryos (red arrows). (I–K) Alcian blue/Alizarin red section histology showed that areolar mineralized tissue constituted the only centra layer in little skate embryos (I,J), whereas an outer mineralized centra layer was present in 6.5 cm DW juveniles (K). (L) Tissue mineral density (TMD) of centra in ratfish was similar to those of stage 33 embryos and 6.5 cm DW juveniles of little skate. Abbreviation: A, anterior; Ab, Alcian blue; Ar, Alizarin red; bd, basidorsal; C, centrum; HA, haemal arch; L, lateral; M, medial; NA, neural arch; OL, outer layer; P, posterior. * indicates statistically significant comparison ($p < 0.05$). Scale bars: as indicated.

et al., 2019 [\[1\]](#); Kemp and Westrin, 1979 [\[2\]](#); Maisey, 2013 [\[3\]](#); Marrama et al., 2019 [\[4\]](#); Seidel et al., 2016 [\[5\]](#); Wilga and Ferry, 2015 [\[6\]](#)), but trabecular tesserae were recently characterized in skates and rays (Atake et al., 2019 [\[7\]](#); Jayasankar et al., 2020 [\[8\]](#)).

Two main qualities of this paper go a long way in revealing features of the ancestral chondrichthyan endoskeleton. First, we clearly identified discrete character states of the extant chondrichthyan endoskeleton (**Fig. 7** [\[9\]](#)), helping to unify terminology of important features for the growing chondrichthyan research community. Second, we presented extensive, side-by-side comparative data across selected representatives of extant chondrichthyans, two elasmobranch species (little skate and small-spotted catshark) and one chimaera (spotted ratfish). The inclusion of a representative of chimaeras is another critical aspect of this paper, because the other chondrichthyan subclass Holocephali is a severely understudied clade that must be considered when inferring ancestral chondrichthyan traits.

Our data provided a critical comparison of character states of the extant chondrichthyan endoskeleton across various species (**Fig. 7** [\[9\]](#)), especially since previous characterization of skeletal mineralization in other adult chimaeridae neither focused on segmented neural arches nor clarified histological zones of tesserae (Berio et al., 2021 [\[10\]](#); Debais-Thibaud, 2018 [\[11\]](#); Pears et al., 2020 [\[12\]](#); Seidel et al., 2020 [\[13\]](#)). Morphological and histological features of bone-like tissues in neural arches or tesseral cap zones were present in little skate and catshark, but absent in ratfish. While these data refute the hypothesis that bone-like tissues generally are a symplesiomorphic (shared ancestral) feature of extant chondrichthyans, they further support the hypothesis that neural arch perichondral bone-like tissue is a synapomorphic (shared derived) character of elasmobranchs (**Fig. 7** [\[9\]](#); Atake et al., 2019 [\[7\]](#); Berio et al., 2021 [\[10\]](#); Maisey et al., 2021 [\[14\]](#)). On the other hand, similar histological features of centra were observed in little skate, catshark, and ratfish, and histological features of multiple-ringed centra in other chimaera should be determined (**Fig. 5D-I** [\[9\]](#); **Fig. 7** [\[9\]](#); Didier, 1995 [\[15\]](#)). Since stem chondrichthyans did not typically mineralize their centra, areolar mineralized tissue is likely a synapomorphic character of crown chondrichthyans (**Fig. 7** [\[9\]](#); Maisey et al., 2021 [\[14\]](#); Miles, 1970 [\[16\]](#)).

In what appears to be a newly-defined symplesiomorphy of crown chondrichthyans, trabecular mineralization occurs in many endoskeletal regions in several elasmobranch and chimaera species, including some fossil chondrichthyans (**Fig. 3** [\[9\]](#); **Fig. 7** [\[9\]](#); Atake et al., 2019 [\[7\]](#); Coates et al., 2018 [\[17\]](#); Frey et al., 2019 [\[18\]](#); Jayasankar et al., 2020 [\[8\]](#); Maisey et al., 2021 [\[14\]](#)). While trabecular mineralization always occurred near the surface of cartilage, the exact mineralization pattern varied among extant chondrichthyans. Little skate had arrayed patterns of discrete trabecular units, similar to the repeating polygonal units of traditional tesserae, supporting their description as trabecular tesserae (**Fig. 7** [\[9\]](#)). On the other hand, trabecular mineralization in catshark and ratfish did not show arrayed patterns of discrete units. While this non-arrayed trabecular mineralization pattern had not been characterized before in extant chondrichthyans, it is also evident in recent images published from the chimaeras *Callorhinchus milii* and *Chimaera monstrosa* (Pears et al., 2020 [\[12\]](#); Seidel et al., 2020 [\[13\]](#)). To distinguish this pattern from the regular, arrayed patterns of polygonal and trabecular tesserae, we propose the term “non-tesseral trabecular mineralization” as a new plesiomorphic (ancestral) character of chondrichthyans (**Fig. 7** [\[9\]](#); Maisey et al., 2021 [\[14\]](#)). Interestingly, some fossil chondrichthyans also appear to display non-tesseral trabecular mineralization in their endoskeletons (Coates et al., 2018 [\[17\]](#); Frey et al., 2019 [\[18\]](#); Ørvig, 1951 [\[19\]](#)).

Cap and body zones are histological terms that traditionally describe chondrichthyan tesserae (Dean and Summers, 2006 [\[20\]](#); Kemp and Westrin, 1979 [\[2\]](#)), but data from adult samples of little skate, catshark, and ratfish argued that the body zone is the only histological zone of endoskeletal mineralization shared by extant chondrichthyans. Published histological data of tesserae are few and limited to sharks, skates, and rays, where cap and body zones have been reported (Atake et al., 2019 [\[7\]](#); Berio et al., 2021 [\[10\]](#); Eames et al., 2007 [\[21\]](#); Enault et al., 2015 [\[22\]](#); Kemp and Westrin, 1979 [\[2\]](#);

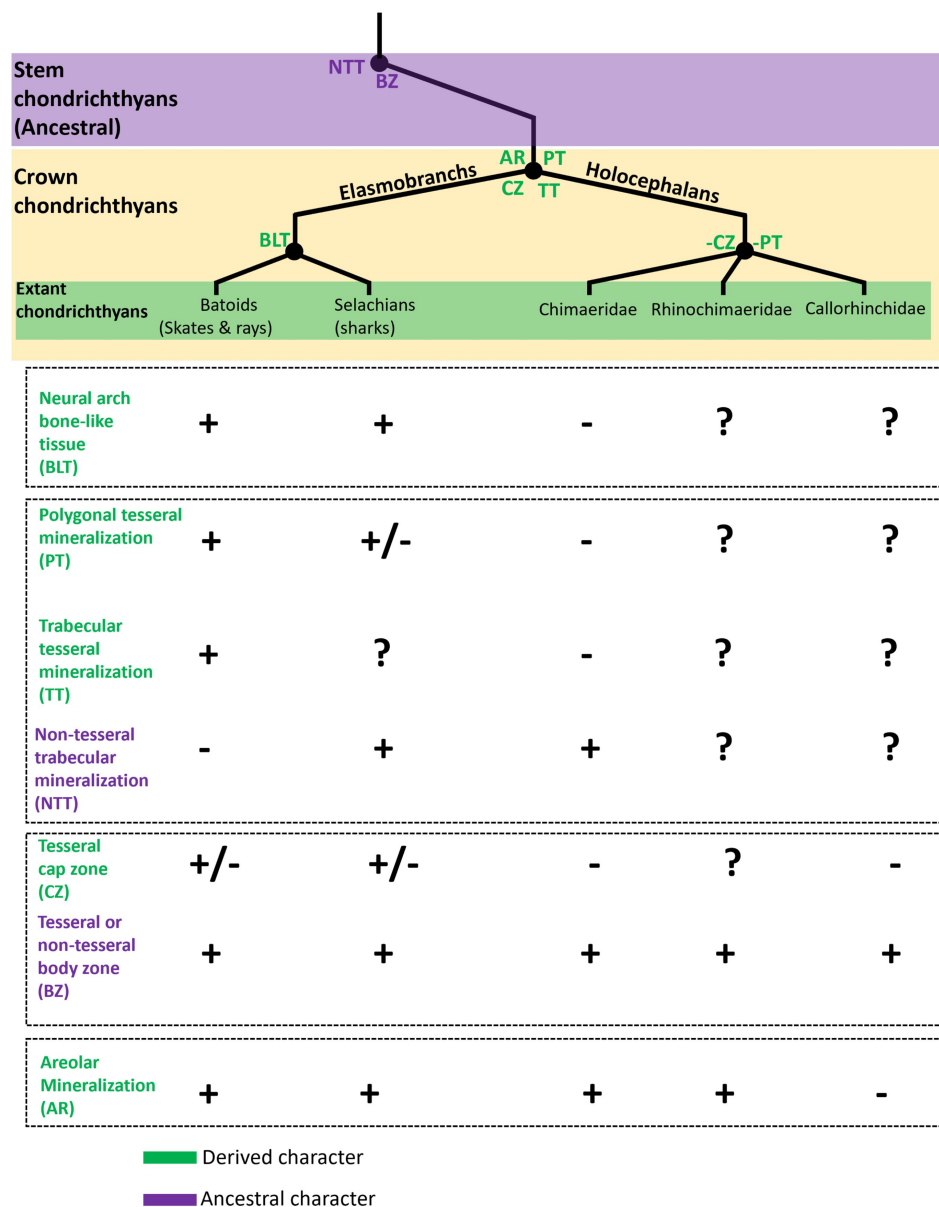


Figure 7.

Character states of mineralized tissues in various chondrichthyan clades suggest shared and derived features. The absence of neural arch bone-like tissue (BLT) in holocephalans and ancestral crown chondrichthyans (from fossils) would further support the conclusion that neural arch bone-like tissue is a synapomorphic (shared derived) character of elasmobranchs. Polygonal (PT) and trabecular (TT) tesseral mineralization patterns appear to be absent in extant holocephalans and stem chondrichthyans, but their presence in some elasmobranchs and some fossil holocephalans suggest that these tesseral mineralizations are synapomorphic characters of crown chondrichthyans. Non-tesseral trabecular mineralization (NTT) is a symplesiomorphic (shared ancestral) character of stem and crown chondrichthyans, because it is pervasive in ancestral and extant chondrichthyans. The histological cap zone in some elasmobranchs, along with the inference that polygonal tesserae in fossil holocephalans means that they had cap zones, suggests that the cap zone is also a synapomorphic character of crown chondrichthyans. The presence of a histological body zone in either tesseral or non-tesseral mineralized tissues of stem and crown chondrichthyans suggests that the body zone is a symplesiomorphic character of all chondrichthyans. Areolar mineralized tissue is present in elasmobranchs and holocephalans, but its absence in stem chondrichthyans suggests that it is a synapomorphic character of crown chondrichthyans.

Pears et al., 2020 [↗](#); Seidel et al., 2017 [↗](#)). While Fast green staining and birefringent fibers marked the cap zones in both polygonal and trabecular tesserae in little skate, non-tesseral trabeculae in catshark and ratfish did not have discernible cap zones. In contrast to commonly-accepted knowledge, these catshark data suggest that even sharks do not always have the cap zone. Taken alongside recent published data (Maisey et al., 2021 [↗](#); Pears et al., 2020 [↗](#); Seidel et al., 2020 [↗](#)), no living species of chimaera likely has cap zones. On the other hand, Safranin O staining and Col2 immunofluorescence showed that histological body zones generated the mineralized endoskeletal cartilage of little skate, catshark, and ratfish. In total, these data suggest that the ancestral stem chondrichthyan generated most of its endoskeletal mineralization with histological body zones, although as the next paragraph highlights, cap zones might have been lost secondarily in chimaera and some shark species (Fig. 7 [↗](#)).

Digitally segmented 3D regions of micro-CT renderings showed, for the first time, exact spatial correlations between histological zones and mineralization patterns, potentially impacting histological interpretations of fossils. Strikingly, large and laterally extensive cap zones were the basis of the traditional polygonal tesseral mineralization pattern in little skate. Accordingly, similar polygonal tesserae recently described in fossil holocephalans likely have cap zones in addition to body zones and a trabecular tesseral mineralization underlying the polygonal mineralization pattern, and these might have been lost secondarily during holocephalan evolution (Fig. 3E [↗](#); Fig. 4 [↗](#); Fig. 7 [↗](#); Atake et al., 2019 [↗](#); Pears et al., 2020 [↗](#)). Careful description of polygonal tesserae in fossil elasmobranchs and holocephalans can clarify this point. Two other correlations were identified here in body zones. First, spokes were located in body zones. Second, spoke and non-spoke regions of the body zone underlie the trabecular mineralization patterns, regardless of whether these were organized as arrayed tesserae or not. Spokes appeared much more spatially organized in polygonal tesserae and trabecular tesserae than in the non-tesseral trabecular mineralization pattern. By extension of the body zone correlation with trabecular mineralization, we propose that trabecular mineralization of some fossil elasmobranchs and holocephalans like *Palaeobates polaris* and *Cladoselache* derives solely from body zones (Fig. 3G,H [↗](#); Frey et al., 2019 [↗](#); Ørvig, 1951 [↗](#)). Given such dramatic variation in extant and fossil chondrichthyans, cellular and molecular mechanisms underlying the evolution of tesseral and non-tesseral mineralization patterns is a wide open field of future study.

Finally, three independent features of the adult ratfish endoskeleton argued that chimaeras might have paedomorphic skeletal development, compared to elasmobranchs. First, tissue mineral density (TMD) was significantly lower in a number of adult ratfish skeletal elements, compared to adult little skate and catshark. Reduced TMD might reflect paedomorphism, because as mineralized skeletal tissues develop, their TMD tends to increase (Forbes, 1976 [↗](#)). Indeed, the TMD of adult ratfish centra was comparable to the TMD of embryonic little skate centra. Second, the morphology of adult ratfish mineralized centra was comparable to the morphology of embryonic little skate mineralized centra. Mineralized centra in chimaeras were thought to be unconstricted (Didier, 1995 [↗](#); Gadow and Abbott, 1895 [↗](#)), but micro-CT renderings of ratfish centra showed slight constriction, which was like those of centra in little skate embryos. Finally, even histological similarities of centra in little skate embryos and adult ratfish were discovered, since both had elongate cell lacunae organized in concentric lamellae only in the mineralized middle layer of the centrum. These findings independently supported a previous assertion that the perichordal sheath in adult chimaeras remained at a stage corresponding to that of embryonic elasmobranchs (Gadow and Abbott, 1895 [↗](#)), but also extend it to apply to chimaera skeletal development generally.

Reduced skeletal mineralization in chimaeras should be investigated further, especially since the only published full chondrichthyan genome was from *Callorhinchus milii* (Callorhinchidae) (Venkatesh et al., 2014 [↗](#)), and compared to other chimaera families, callorhinchidae appear to demonstrate less skeletal mineralization. For example, callorhinchidae are the only chimaera family that do not mineralize their centra (Fig. 7 [↗](#); Didier, 1995 [↗](#); Gadow and Abbott, 1895 [↗](#)).

The genetic basis of skeletal mineralization in vertebrates is linked to the secretory calcium-binding phosphoprotein (SCPP) family of genes (Kawasaki et al., 2004 [DOI](#); Kawasaki and Weiss, 2003 [DOI](#)), none of which is present in the genome of *Callorhinchus milii* (Venkatesh et al., 2014 [DOI](#)). Elasmobranch data are sparse, but catshark ameloblasts express an SCPP gene (Leurs et al., 2022 [DOI](#)). Like elasmobranchs, the basal ray-fin fish order Acipenseriformes (sturgeons, paddlefish) also have a predominantly cartilaginous skeleton, bone-like tissues, and SCPP genes (Cheng et al., 2021 [DOI](#); Leprevost et al., 2017 [DOI](#); Mikami et al., 2022 [DOI](#); Warth et al., 2017 [DOI](#)). Future studies should carefully assess the potential role of SCPP genes in the mineralization of diverse chondrichthyan mineralized tissue types.

Materials and Methods

Specimens

All samples were approved for use under the University of Saskatchewan ethical protocol (AUP 20130092). Vertebrae from three samples of adult small-spotted catshark with total lengths of 31 cm were provided by the University of Montpellier Aquarium (Planet Ocean Montpellier). Adult, juvenile, and embryonic samples of the little skate were obtained from Marine Biological Labs (Falmouth, MA, USA). Four samples of adult little skate with total lengths (TL) ranging from 43.5 cm – 47.5 cm were sampled (SupTable 1). Little skate juveniles (n=7) were staged by measuring their TL and disc widths (DW) and ranged from 5.5 cm DW, 10 cm TL to 6.5 cm DW, 11 cm TL (SupTable 1; Stehmann, 2002 [DOI](#)). The average DW and TL of stage 32 embryos (n=6) and stage 33 embryos (n=6) were 3.2 cm DW, 7.5 cm TL and 3.5 cm DW, 8 cm TL, respectively (SupTable 1; Maxwell et al., 2008 [DOI](#); Vazquez et al., 2020 [DOI](#)). Five samples of adult spotted ratfish with total lengths ranging from 28.5 cm to 45 cm were captured by deep-water trawl in the San Juan Islands, WA (SupTable 1).

Regions of interest, such as ceratohyal, synarcual, and precaudal and caudal vertebrae, were dissected from the little skate, catshark, and ratfish samples (SupTable 1). Dissected tissues were preserved by fixation in 4% paraformaldehyde in PBS (pH 7.4) and dehydrated through a graded ethanol series. Tissues for section histology were not demineralized before sectioning. Tissues were embedded in optimum cutting temperature compound (Tissue Tek, Torrance, CA, United States), and serial tissue sections of 10 µm thickness were made with a Cryostar NX50 cryostat (Fisher Scientific, United States).

Micro-CT imaging and data processing

Desktop micro-CT imaging of little skate and ratfish tissues was done using SkyScan 1172 desktop microtomograph (Bruker SkyScan, Kontich, Belgium). Desktop micro-CT projections were acquired using a 0.5 mm aluminium filter at 40 kV and 250 µA, 100 ms exposure time, and 10 µm voxel resolution, and reconstructed using NRecon (Bruker SkyScan, Kontich, Belgium). An anterior region of the ratfish was cut into parts before scanning (Fig. 1A [DOI](#)). Scans from the different parts were later stitched together using Dragonfly v2021.1 (Object Research Systems, Canada). Desktop imaging of catshark samples was performed as previously described (Berio et al., 2021 [DOI](#)). Tissue mineral density (TMD) was quantitated on desktop micro-CT images using CTAnalyzer v1.16 (CTAn, Bruker SkyScan, Kontich, Belgium) as previously described (Atake et al., 2019 [DOI](#)).

Synchrotron radiation (SR)-based micro-CT imaging was done on the Biomedical Imaging and Therapy-Insertion Device 05ID-2 (BMIT-ID) line at the Canadian Light Source. SR micro-CT projections were acquired with a 28 keV photon energy, 1.44 µm voxel resolution, 1s exposure time, and a sample to detector distance of 9 cm. Reconstruction of SR micro-CT projections and phase retrieval was done using UFO-KIT software (<https://github.com/ufo-kit/ufo-core.git>). A

delta/beta ratio of 200 was applied during phase retrieval. 3D rendered volumes and 2D virtual slices of reconstructed data were generated with Amira 6.0 (FEI Group, USA). 3D segmentation and color-coding of morphological features were done using segmentation editor tools in Amira.

Histological and immunofluorescence assays

Tissue sections were stained as described with Safranin O (Ferguson et al., 1998 [↗](#)), picrosirius red (Junqueira et al., 1978 [↗](#)), or Milligan's Trichrome (Ashique et al., 2022 [↗](#)). Alcian blue/Alizarin red staining was done using a modified acid-free protocol (Eames et al., 2011 [↗](#)). Briefly, tissues sections were washed with 100 mM Tris pH 7.5/10 mM MgCl₂, stained with 0.04% Alcian blue/70%EtOH/10mM MgCl₂ pH 7.5, taken through graded EtOH series (80% EtOH/100 mM Tris pH 7.5/10 mM MgCl₂; 50% EtOH/100 mM Tris pH 7.5; 25% EtOH/100 mM Tris pH 7.5), stained with 0.1% Alizarin red/0.1 % KOH pH 7.5, de-stained with two washes of 0.1% KOH, and dehydrated through 25% EtOH, 50% EtOH, 80% EtOH, and 100% EtOH series before coverslipping. Tissue sections were demineralized using 5% EDTA for 10 mins before staining with Safranin O to improve staining of mineralized cartilage.

To minimize the chances of tissues falling off slides, tissue sections for immunofluorescence were incubated at 55°C for 15 mins before treatments and then washed with PBST (1xPBS/0.5x triton-X) between subsequent treatments. Tissues were treated separately with trypsin (0.1% in 5%EDTA/1xPBS) and hyaluronidase (0.5% in 1xPBS/0.5x triton-X) at 37°C for 15 mins each. The tissues were blocked with 4% goat serum/2% sheep/1xPBS for 1 hr, and labelled with Col2 antibody (1:100, II-II6B3, Hybridoma Bank) overnight at 4°C. Secondary antibody labelling was done using 488-conjugated goat anti-mouse antibody (1:1000, A32723 ThermoFisher Scientific) for 3 hours.

Quantitative and statistical analyses

Statistical analyses of TMDs were performed using SPSS V.22 (SPSS). Shapiro–Wilk test was used to test for normal distribution of data. To assess differences among means, one-way analysis of variance (ANOVA) was used followed by Tukey HSD or Games-Howell post hoc analyses, depending upon whether homogeneity of variance assumption was met or not, respectively.

Data availability

Raw data from microCT projections and quantitative measurements have been deposited at Dryad (DOI: 10.5061/dryad.crjdfn3b9) and can be accessed at <https://datadryad.org/stash/share/gnKH8kfRoKdHRE9rFGDausGzBZ1VTuCK3DB5gRPkrnp> [↗](#)

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Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany

Reviewer #1 (Public Review):

Summary:

It seems as if the main point of the paper is about the new data related to rat fish although your title is describing it as extant cartilaginous fishes and you bounce around between the little skate and ratfish. So here's an opportunity for you to adjust the title to emphasize ratfish is given the fact that leader you describe how this is your significant new data contribution. Either way, the organization of the paper can be adjusted so that the reader can follow along the same order for all sections so that it's very clear for comparative purposes of new data and what they mean. My opinion is that I want to read, for each subheading in the results, about the the ratfish first because this is your most interesting novel data. Then I want to know any confirmation about morphology in little skate. And then I want to know about any gaps you fill with the cat shark. (It is ok if you keep the order of "skate, ratfish, then shark, but I think it undersells the new data).

Strengths:

The imagery and new data availability for ratfish are valuable and may help to determine new phylogenetically informative characters for understanding the evolution of cartilaginous fishes. You also allude to the fossil record.

Opportunities:

I am concerned about the statement of ratfish paedomorphism because stage 32 and 33 were not statistically significantly different from one another (figure and prior sentences). So, these ratfish TMDs overlap the range of both 32 and 33. I think you need more specimens and stages to state this definitely based on TMD. What else leads you to think these are paedomorphic? Right now they are different, but it's unclear why. You need more outgroups.

Your headings for the results subsection and figures are nice snapshots of your interpretations of the results and I think they would be better repurposed in your abstract, which needs more depth.

Historical literature is more abundant than what you've listed. Your first sentence describes a long fascination and only goes back to 1990. But there are authors that have had this fascination for centuries and so I think you'll benefit from looking back. Especially because several of them have looked into histology and development of these fishes.

I agree that in the past 15 years or so a lot more work has been done because it can be done using newer technologies and I don't think your list is exhaustive. You need to expand this list and history which will help with your ultimate comparative analysis without you needed to sample too many new data yourself.

I'd like to see modifications to figure 7 so that you can add more continuity between the characters, illustrated in figure 7 and the body of the text. Generally Holocephalans are the outgroup to elasmobranchs - right now they are presented as sister taxa with no ability to indicate derivation. Why isn't the catshark included in this diagram?

In the last paragraph of the introduction, you say that "the data argue" and I admit, I am confused. Whose data? Is this a prediction or results or summary of other people's work? Either way, could be clarified to emphasize the contribution you are about to present.

<https://doi.org/10.7554/eLife.94900.1.sa1>

Reviewer #2 (Public Review):

General comment:

This is a very valuable and unique comparative study. An excellent combination of scanning and histological data from three different species is presented. Obtaining the material for such a comparative study is never trivial. The study presents new data and thus provides the basis for an in-depth discussion about chondrichthyan mineralised skeletal tissues. I have, however, some comments. Some information is lacking and should be added to the manuscript text. I also suggest changes in the result and the discussion section of the manuscript.

Introduction:

The reader gets the impression almost no research on chondrichthyan skeletal tissues was done before the 2010 ("last 15 years", L45). I suggest to correct that and to cite also previous studies on chondrichthyan skeletal tissues, this includes studies from before 1900.

Material and Methods:

Please complete L473-492: Three different Micro-CT scanners were used for three different species? ScyScan 117 for the skate samples. Catshark different scanner, please provide full details. Chimera Scncrotron Scan? Please provide full details for all scanning protocols.

TMD is established in the same way in all three scanners? Actually not possible. Or, all specimens were scanned with the same scanner to establish TMD? If so please provide the protocol.

Please complete L494 ff: Tissue embedding medium and embedding protocol is missing. Specimens have been decalcified, if yes how? Have specimens been sectioned non-decalcified or decalcified?

Please complete L506 ff: Tissue embedding medium and embedding protocol is missing. Description of controls are missing.

Results:

L147: It is valuable and interesting to compare the degree of mineralisation in individuals from the three different species. It appears, however, not possible to provide numerical data for Tissue Mineral Density (TMD). First requirement, all specimens must be scanned with the same scanner and the same calibration values. This is not stated in the M&M section. But even if this was the case, all specimens derive from different sample locations and have been preserved differently. Type of fixation, extension of fixation time in formalin, frozen, unfrozen, conditions of sample storage, age of the samples, and many more parameters, all influence TMD values. Likewise the relative age of the animals (adult is not the same as adult) influences TMD. One must assume different sampling and storage conditions and different types of progression into adulthood. Thus, the observation of different degrees of mineralisation is very interesting but I suggest not to link this observation to numerical values.

Parts of the results are mixed with discussion. Sometimes, a result chapter also needs a few references but this result chapter is full of references.

Based on different protocols, the staining characteristics of the tissue are analysed. This is very good and provides valuable additional data. The authors should inform not only about the staining (positive or negative) but also about the histochemical characters of the staining. L218: "fast green positive" means what? L234: "marked by Trichrome acid fuchsin" means what? And so on, see also L237, L289, L291

Discussion

Please completely remove figure 7, please adjust and severely downsize the discussion related to figure 7. It is very interesting and valuable to compare three species from three different groups of elasmobranchs. Results of this comparison also validate an interesting discussion about possible phylogenetic aspects. This is, however, not the basis for claims about the skeletal tissue organisation of all extinct and extant members of the groups to which the three species belong. The discussion refers to "selected representatives" (L364), but how representative are the selected species? Can there be an extant species that represents the entire large group, all sharks, rays or chimeras? Are the three selected species basal representatives with a generalist life style?

Please completely remove the discussion about paedomorphosis in chimeras (already in the result section). This discussion is based on a wrong idea about the definition of paedomorphosis. Paedomorphosis can occur in members of the same group. Humans have paedomorphic characters within the primates, *Ambystoma mexicanum* is paedomorphic within the urodeals. Paedomorphosis does not extend to members of different vertebrate branches. That elasmobranchs have a developmental stage that resembles chimera vertebra mineralisation does not define chimera vertebra centra as paedomorphic. Teleosts have a heterocercal caudal fin anlage during development, that does not mean the heterocercal fins in sturgeons or elasmobranchs are paedomorphic characters.

L432-435: In times of Gadow & Abott (1895) science had completely wrong ideas about the phylogenetic position of chondrichthyans within the gnathostomes. It is curious that Gadow & Abott (1895) are being cited in support of the pedomorphosis claim.

The SCPP part of the discussion is unrelated to the data obtained by this study. Kawaki & WEISS (2003) describe a gene family (called SCPP) that control Ca-binding extracellular phosphoproteins in enamel, in bone and dentine, in saliva and in milk. It evolved by gene duplication and differentiation. They date it back to a first enamel matrix protein in conodonts (Reif 2006). Conodonts, a group of enigmatic invertebrates have mineralised structures but these structures are neither bone nor mineralised cartilage. Catfish (6 % of all vertebrate species) on the other hand, have bone but do not have SCPP genes (Lui et al. 2006). Other calcium binding proteins, such as osteocalcin, were initially believed to be required for mineralisation. It turned out that osteocalcin is rather a mineralisation inhibitor, at best it regulates the arrangement collagen fiber bundles. The osteocalcin $-/-$ mouse has fully mineralised bone. As the function of the SCPP gene product for bone formation is unknown, there is no need to discuss SCPP genes. It would perhaps be better to finish the manuscript with a summary that focuses on the subject and the methodology of this nice study.

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