Coevolution of enamel, ganoin, enameloid, and their matrix SCPP genes in osteichthyans

**HIGHLIGHTS**

- Ganoin emerged in actinopterygians; true enamel arose in sarcopterygians
- Dental enamel, acrodin, and enameloid in actinopterygians are related to ganoin
- SCPP5 evolved in association with ganoin, whereas AMEL evolved with true enamel
- Shifts in SCPP gene expression explain the evolution of hypermineralized tissues

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Coevolution of enamel, ganoin, enameloid, and their matrix SCPP genes in osteichthyans

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SUMMARY
We resolve debate over the evolution of vertebrate hypermineralized tissues through analyses of matrix protein-encoding secretory calcium-binding phosphoprotein (SCPP) genes and phylogenetic inference of hypermineralized tissues. Among these genes, AMBN and ENAM are found in both sarcopterygians and actinopterygians, whereas AMEL and SCPP5 are found only in sarcopterygians and actinopterygians, respectively. Actinopterygian AMBN, ENAM, and SCPP5 are expressed during the formation of hypermineralized tissues on scales and teeth: ganoin, acrodin, and collar enamel in gar, and acrodin and collar enameloid in zebrafish. Our phylogenetic analyses indicate the emergence of an ancestral enamel in stem-osteichthyans, whereas ganoin emerged in stem-actinopterygians and true enamel in stem-sarcopterygians. Thus, AMBN and ENAM originated in concert with ancestral enamel, SCPP5 evolved in association with ganoin, and AMEL evolved with true enamel. Shifts in gene expression domain and timing explain the evolution of different hypermineralized tissues. We propose that hypermineralized tissues in osteichthyans coevolved with matrix SCPP genes.

INTRODUCTION
The vertebrate skeleton is composed principally of cartilage, bone, dentine, enamel, and enameloid (Donoghue et al., 2006; Hall, 2015), all of which are critical to vertebrate adaptations, including protective body armor, an endoskeleton for locomotion, and teeth for feeding (Donoghue and Keating, 2014). As such, mineralized tissues comprise a key innovation underlying much of vertebrate evolutionary success. Among these skeletal tissues, the origin of hypermineralized tissues, enamel, enameloid, and their histological derivatives, remains controversial, partly because their classification in fossils varies among researchers (Friedman and Brazeau, 2010; Schultze, 2016) and partly because genes encoding their matrix proteins are well understood only for true enamel in sarcopterygians. Here we investigate the evolution of various hypermineralized tissues through genomic and developmental analyses, combined with estimation of ancestral states based on data available from living and fossil vertebrates. We draw these disparate approaches together to obtain a holistic understanding of the origin and diversification of vertebrate hypermineralized tissues. Our results reveal evidence for the coevolution of hypermineralized tissues in osteichthyans and their matrix secretory calcium-binding phosphoprotein (SCPP) genes.

RESULTS
Enamel and enameloid have been classified into different types depending on their location and histological characteristics (Figure 1A). Mineralization of enamel and enameloid progresses in organic matrices (Berkovitz and Shellis, 2016) that are subsequently removed as they mature into hypermineralized inorganic tissues (Sasagawa, 1997; Fincham et al., 1999). Enamel grows in a non-collagenous matrix secreted by ameloblasts of epithelial origin (Fincham et al., 1999) and occurs in three main types: (1) true enamel, considered equivalent to mammalian tooth enamel (Smith, 1989); (2) multilayered ganoin (Schultze, 2016) on scales and their derivatives, found only in bichirs andgars among extant clades, as well as in diverse fossil actinopterygians (Sire et al., 2009); and (3) tooth collar enamel, which occurs in actinopterygians, including bichirs, gars, and extinct clades (Smith, 1995; Ishiyama et al., 1999; Sasagawa et al., 2013). Enameloid forms in a collagenous matrix secreted by both inner dental epithelial (IDE) cells and mesenchyme-derived odontoblasts (Poole, 1967), often characterized historically by protruding dentine tubules (Smith, 1995). Enameloid constitutes an acrodin tooth cap in various extant and extinct actinopterygians
Figure 1. Phylogenetic distribution of enamel and enameloid matrix genes, and exon-intron structure of AMEL and SCPP5

(A) Phylogenetic distribution of enamel, enameloid, and their matrix genes. Hypermineralized tissues classified as enamel are denoted by an asterisk. Divergence times are based on previous studies: most sarcopterygian taxa (Hedges and Kumar, 2009), actinopterygian taxa (Near et al., 2012), lungfish-tetrapods and chondrichthyans-osteichthyans (Giles et al., 2017), and agnathans-gnathostomes (Kuraku and Kuratani, 2006).

(B) Exon-intron structure of AMEL and SCPP5. Separate boxes represent exons. Open areas in exons show 5’ and 3’ untranslated regions. The areas encoding the signal peptide and mature protein are shown in yellow and vermilion, respectively. Exon numbers are indicated for human AMEL (AMELX) and bichir SCPP5. Locations encoding phospho-Ser residues (S*) (Kawasaki and Amemiya, 2014) and Pro-Xaa-Yaa (Xaa and Yaa represent any amino acids; PXY) repeats are illustrated within exon boxes. Regions encoding the three modules, the N-terminal aromatic residue-rich region (YY), the Pro/Gln-rich core region containing uninterrupted PXY repeats (P/Q), and the C-terminal hydrophilic region (DE), are indicated below exon boxes. Scale bar, 100 nucleotides (nt). See Figure S1A for details.

(Shellis and Miles, 1974; Sasagawa et al., 2013; Schultze, 2016). Tooth collar enameloid occurs in teleosts (Shellis and Miles, 1974; Sasagawa, 1988; Smith, 1995). Enameloid is also found on the dental and dermal skeleton in chondrichthians (including acanthodians), as well as in extinct jawed and jawless stem-gnathostomes (Donoghue et al., 2006; Rücklin et al., 2011; Keating et al., 2015).

Enamel and enameloid matrix genes in sarcopterygians and actinopterygians

The amelogenin (AMEL), ameloblastin (AMBN), and enamelin (ENAM) genes encode the principal enamel matrix proteins (Fincham et al., 1999) in tetrapods (presumably also coelacanth [Kawasaki and Amemiya, 2014]), whereas SCPP5 is thought to encode an enameloid matrix protein in Fugu rubripes (fugu) and Danio rerio (zebrafish) (Kawasaki et al., 2005, 2009). AMEL has been found only in sarcopterygians and SCPP5 only in actinopterygians (Figure S1A) (Qu et al., 2015; Braasch et al., 2016; Kawasaki et al., 2017). AMBN and ENAM are also found in actinopterygians, including Lepisosteus oculatus (referred to below as “gar”) and zebrafish (Figures S1B and S1C) (Braasch et al., 2016; Kawasaki et al., 2017). All four genes belong to the SCPP gene family that arose by gene duplication (Kawasaki and Weiss, 2003; Kawasaki et al., 2017). It is notable that no SCPP genes have been identified in chondrichthyans (Figure 1A) (Venkatesh et al., 2014; Enault et al., 2018).

We identified amel and ambn in Lepidosiren paradoxa (lungfish); ambn, enam, and scpp5 in Polypterus senegalus (referred to below as “bichir”) and various teleosts; and ambn in Acipenser sinensis (sturgeon; Figure S1); AMEL was not identified in actinopterygians. All three modules characteristic of tetrapod
and coelacanth amelogenins (YY-, P/Q-, and DE-rich regions; Figures 1B and S1A) (Toyosawa et al., 1998; Fincham et al., 1999) were detected in proteins encoded by amel in L. paradoxa and scpp5 in bichir and gar. Nevertheless, in most sarcopterygians these modules are encoded by five exons in AMEL, but twelve exons in bichir and gar scpp5 genes (Figure 1B). Furthermore, AMEL differs from SCPP5 in genomic location, exon-intron organization, and exons encoding phospho-Ser residues (Figure S1A). These data support interpretation independent origin of AMEL and SCPP5. Teleosts do not possess enamel, and their scpp5 genes lack two of these three modules (P/Q and DE; Figures 1B and S1A).

**Expression of ambn, enam, and scpp5 in teeth and scales of gar and teeth of zebrafish**

In gar, expression of ambn, enam, and scpp5 was detected during tooth formation in IDE cells, initially during acrodin matrix formation (matrix formation stage of enameloid; Figures 2A–2D) and then during collar enamel matrix formation (secretory stage of enamel; Figures 2E–2H) (Sasagawa and Ishiyama, 2005; Sasagawa et al., 2008). During scale formation, expression of ambn, enam, and scpp5 was detected in inner ganoin epithelial (IGE) cells that secrete the ganoin matrix on scales (secretory stage; Figures 2I–2L). We detected no other expression domains of these genes (Figure S2A). Given the limited expression domain of these genes in gar skin, their relative expression levels in IGE cells can be determined by RNA sequencing (RNA-seq) analysis of the skin (Qu et al., 2015; Braasch et al., 2016). Expression of scpp5 was the highest among these genes and among all SCPP genes (Table S1).
In zebrafish, expression of *ambn* and *enam* was detected in IDE cells in the matrix formation stages of acrodin and collar enameloid (Figures 2M–2O and S2A); *enam* was also expressed in odontoblasts, but we detected no significant expression of *ambn* or *enam* in bone cells (Figures 2N and 2O). Expression of *enam* in odontoblasts appears to initiate during acrodin matrix formation and continue throughout collar enameloid and dentine matrix formation (Figures 2O and S2A) (Shellis and Miles, 1974). Results of our expression analysis are summarized in Figure 2P.

**Distribution of gar Scpp5 in acrodin and collar enamel on teeth and in ganoin on scales**

The results of our in situ hybridization analysis for gar scpp5 in acrodin and collar enameloid (Figures 2M–2O and S2A); *enam* was also expressed in odontoblasts, but we detected no significant expression of *enam* or *ambn* in bone cells (Figures 2N and 2O). Expression of *enam* in odontoblasts appears to initiate during acrodin matrix formation and continue throughout collar enameloid and dentine matrix formation (Figures 2O and S2A) (Shellis and Miles, 1974). Results of our expression analysis are summarized in Figure 2P.

Since our optical IHC analysis detected specific distributions of Scpp5 in the matrix of developing teeth and scales, we furthered IHC analysis using transmission electron microscopy (TEM). The results revealed an association of Scpp5 with the edge of collagen fibrils in the mineralization stage of acrodin formation (Figure 4A). In the developing collar enamel, Scpp5 was associated with electron-dense fibrils (Figure 4B) postulated to form as organic sheaths surrounding slender crystals (Warshawsky, 1989). Scpp5 was also detected in the underlying dentine near the border with collar enamel (Figure 4C), corroborating optical microscopic observations (Figure 3D). As in collar enamel, most Scpp5 signals in developing ganoin were associated with electron-dense fibrils (Figures 4D and 4E), especially along fibril edges, suggesting interactions of Scpp5 with minerals or mineral-associated organic molecules. Weak but significant signals...
were also detected in bone underlying scale ganoin, but not in deeper regions (Figure 4F), as in dentine underlying collar enamel (Figure 4C).

**Reconstructing ancestral states of hypermineralized tissues**

We explored the evolution of acrodin, enameloid, enamel, and ganoin by reconstructing the ancestral states using data from living and fossil taxa (Table S2), stochastic character state mapping (Huelsenbeck et al., 2003), and phylogenetic hypotheses in which (1) Guiyu, Achoania, and Psarolepis (GAP clade) are constrained to stem-osteichthyans (King et al., 2017; Lu et al., 2017) and (2) these taxa are resolved as stem-sarcopterygians (Lu et al., 2016; Qiao et al., 2016; Choo et al., 2017) (Figures S3A and S3B).

Our results indicate that acrodin evolved in the actinopterygian stem-lineage (*Cheirolepis* and more crown-ward taxa); its presence in *Ligulalepis* is a consequence of convergence or the isolated tooth from which these data derive does not belong to *Ligulalepis* (Figures 5A, 5B, S3C, and S3D). Dental enameloid evolved late in the chondrichthyan stem-lineage (*Pucapampella* or more crown-ward taxa; Figures 5A, 5B, S3E, and S3F); the presence of dental enameloid in acanthodians is convergent. Dermal enameloid was resolved as primitive to the chondrichthyan total-group and, although it is also present in ostracoderms (Donoghue and Sansom, 2002; Keating et al., 2015), it is inferred absent from the dermal skeleton of stem-os teichthyans (Figures 5C, 5D, S3G, and S3H). Thus, dermal enameloid was lost in placoderms (Giles et al., 2013) and evolved convergently in stem-chondrichthyans after the divergence of *Ramirosuarezia* (Figures S3G and S3H). Taken together, these results suggest that, in both the dental and dermal skeletons, osteichthyan enameloid originated independently from chondrichthyan enameloid (but see below).

Our estimates of ancestral states were invariant to whether the GAP clade is resolved as stem-osteichthyan or stem-sarcopterygian (Figure 5). Dental enamel evolved early in the sarcopterygian stem-lineage (*Onychodus* and more crown-ward taxa), arising convergently as collar enamel in non-teleost actinopterygians (bichir and gar; Figures 5A, 5B, S3I, and S3J). Dermal enamel evolved deep in the osteichthyan stem-lineage (*Andreolepis* and more crown-ward taxa), was lost among early stem-actinopterygians (*Cheirolepis* and more crown-ward taxa), but retained in crown-sarcopterygians (Figures 5C, S3K, and S3L). Ganoin evolved among early stem-actinopterygians (*Cheirolepis* and more crown-ward taxa; Figures 5C, 5D, S3M, and S3N); the ganoin-like (Ørvig, 1978; Schultze, 2016) overlapping enamel in *Ligulalepis* (Schultze and Mårs, 2004; Schultze, 2014) is resolved as convergent. If dermal enamel and ganoin are considered homologous, consistent with the expression patterns of *AMBN* and *ENAM* in both sarcopterygian enamel and ganoin, the ancestral tissue evolved deep in the osteichthyan-stem (*Andreolepis* and more crown-ward...
taxa; Figures 5C, 5D, S3O, and S3P). Given the absence of SCPP genes in chondrichthyans, early enamel matrix SCPP genes likely evolved in concert with a prototypic enamel in stem-osteichthyans, from which true enamel and ganoin arose, respectively, in sarcopterygians and actinopterygians, as discussed below (Figure 1A).

Dermal enameloid is present in stem-gnathostomes, early members of placoderm plesia, and chondrichthyans (Giles et al., 2013). It has been hypothesized that enamel replaced enameloid through shifts in the timing of ameloblast and odontoblast activity (Smith, 1992, 1995). Ancestral state estimation provides support for this switch in a combined coding of dermal enamel, ganoin, and enameloid (Figures 5C, 5D, S3Q, and S3R). The initial enamel matrix SCPP gene may have originated in stem-gnathostomes and may have subsequently been lost in stem-chondrichthyans. As we discuss below, however, it is more likely that the enamel matrix SCPP genes are primitively absent from chondrichthyans and were never involved in dermal enameloid development. The origin of AMBN and ENAM in stem-osteichthyans may be invoked in the developmental evolution of enamel from an ancestral dermal enameloid (Donoghue and Sansom, 2002; Donoghue et al., 2006; Keating et al., 2015).

**DISCUSSION**

**Similar formation of ganoin and collar enamel, and of acrodin and collar enameloid**

We detected a similar expression pattern of gar scpp5, ambn, and enam in secretory-stage IGE cells and the uniform distribution of gar Scpp5 in the ganoin matrix (Figures 2J–2L, 3E, and 3F), which suggests that all these three genes encode ganoin matrix proteins. During collar enamel formation, expression of scpp5, ambn, and enam in IDE cells (Figures 2F–2H) and the uniform distribution of Scpp5 in the collar enamel

![Figure 5. Reconstructing ancestral states of hypermineralized tissues](image-url)
matrix (Figures 3C and 3D) were also detected. These results imply that ganoin and collar enamel form in a similar manner. Thus, collar enamel of gar is better described as collar ganoin, which corroborates the hypothesis obtained by histological studies of gars and many fossil actinopterygians (Ørvig, 1978; Schultze, 2016).

Acrodin forms below the basal lamina (BL) that originally separates odontoblasts from IDE cells (Smith, 1995). During acrodin formation in gar, odontoblasts retreat from the BL and secrete the bulk of the collagenous matrix (Sasagawa and Ishiyama, 2005), similar to dentine formation, while IDE cells secrete Scpp5, and presumably also Ambn and Enam, as suggested by the expression of ambn and enam in matrix formation-stage IDE cells. Thus, the region distal to the BL is formed principally by odontoblasts, whereas the contribution of IDE cells is large in the BL-proximal region (we observed the distribution of Scpp5 only near the outer surface; Figures 3A and 3B). This result suggests that gar acrodin forms as a dentine-ganoin composite, supporting the hypothesis for acrodin formation in teleosts (Shellis and Miles, 1974). Association of Scpp5 with collagen in the acrodin matrix during mineralization (Figure 4A) probably affects the mineralization and/or maturation processes, as previously inferred for teleosts (Shellis and Miles, 1974).

During acrodin formation in zebrafish, ambn and enam are expressed in IDE cells and enam are additionally expressed in odontoblasts. Similar to zebrafish enam, zebrafish and fugu scpp5 genes are also expressed in both IDE cells and odontoblasts (Kawasaki et al., 2005; Kawasaki, 2009), unlike their gar orthologs (Figure 2P). Although the expression of these genes suggests conservation of acrodin in gar and zebrafish, expression of zebrafish scpp5 and enam and fugu scpp5 in odontoblasts implies a modification of acrodin. Because ENAM is expressed primarily in cells of epithelial origin during the formation of hypermineralized tissues in gar and sarcopterygians, the modification of acrodin is inferred to have occurred in teleosts. In zebrafish, a similar expression pattern of ambn, enam, and scpp5 in IDE cells and odontoblasts was also detected during collar enameloid formation (Figure 2P) (Kawasaki, 2009). These results suggest that acrodin and collar enameloid form in a similar matrix in zebrafish and support the inference, obtained by studying various teleosts, that acrodin and collar enameloid are homologous, formed by both IDE cells and odontoblasts in a similar manner (Shellis and Miles, 1974; Sasagawa and Ishiyama, 1988).

**Coevolution of hypermineralized tissues and SCPP genes**

Our results suggest that enameloid evolved independently in chondrichthyan species and actinopterygians. Although it is difficult to distinguish enameloid of chondrichthyan species from enameloid of actinopterygians by histological characteristics (Reif, 1979), independent evolution of these tissues is supported by their different mineralization processes. In various actinopterygians, mineralization of enameloid initiates in matrix vesicles, and fine crystals accumulate along collagen fibrils, similar to mineralization of bone and dentine (Shellis and Miles, 1976; Sasagawa, 1988, 1997; Sasagawa et al., 2019). By contrast, mineralization of enameloid in various chondrichthyans begins in tubular vesicles, which are not found in osteichthyans, and no crystals concentrate along fibrillar structures (Sasagawa, 1998, 2002). The unique mineralization process of chondrichthyan enameloid reinforces the idea that this tissue evolved independently of SCPP genes (Kawasaki et al., 2017).

In gar, scpp5 and presumably also ambn and enam encode ganoin matrix proteins, whereas AMEL, AMBN, and ENAM encode true enamel matrix proteins in sarcopterygians. The difference in matrix proteins of ganoin in gar and true enamel in sarcopterygians supports histological classification of true dental enamel in most stem- and crown-sarcopterygians and ganoin on scales in total-group actinopterygians (Qu et al., 2015). AMEL is found only in sarcopterygians; SCPP5 only in actinopterygians (Figure S1). Gar scpp5 shows the highest expression level among all SCPP genes in the skin during ganoin formation (Table S1), reminiscent of AMEL that encodes the most abundant enamel matrix protein (Fincham et al., 1999). Sarcopterygian AMEL genes and gar and bichir scpp5 genes encode a similar modular structure, which is not encoded by scpp5 in teleosts that do not possess enamel (Figure 1). Furthermore, expression of bichir scpp5 was confirmed in the skin during ganoin formation (Figure S1A). These results suggest that sarcopterygian AMEL genes and gar and bichir scpp5 genes have overlapping functions and that either AMEL or scpp5 is sufficient for these overlapping functions during the formation of true enamel and ganoin. We thus assume that AMEL evolved in concert with true enamel and SCPP5 evolved with ganoin.

Given the narrow expression domains and timings of AMBN and ENAM, similar spatiotemporal expression patterns of these genes during the formation of true enamel in sarcopterygians and ganoin in gar imply an
The evolutionary relationship of these two tissues, rather than independent and coincidental employment of AMBN and ENAM in sarcopterygian true enamel and ganoin. Since sarcopterygians and gar phylogenetically bracket sarcopterygians and actinopterygians (Figure 1A) (Witmer, 1995), we hypothesize that both AMBN and ENAM were expressed during the formation of ancestral enamel (see below) in the most recent common ancestor of sarcopterygians and actinopterygians. The evolutionary relationship of true enamel and ganoin in gar and many fossil actinopterygians is supported by the common rod-like arrangement of crystallites, known as the protoprismatic microstructure (Ørvig, 1978; Smith, 1989; Sasagawa et al., 2016). A unique mineralization process of true enamel and ganoin in gar and bichir also supports their evolutionary relationship. During the formation of true enamel and ganoin, enamel ribbons form the mineralization front along the distal membrane of ameloblasts or IGE cells (Sire, 1995; Simmer et al., 2010). The close relationship of true enamel and ganoin suggests their homology: either one tissue derived from the other or both derived from an ancestral enamel.

Enamel and ganoin are considered homologous (Sire et al., 1987; Sasagawa et al., 2013; Qu et al., 2015), and the ancestral character estimation analysis assuming this (Figures 3C and 5D) is phylogenetically congruent, requiring an ancestral dermal hypermineralized tissue in stem-osteichthyan. Within the context of SCPP gene evolution, either AMEL or SCPP5 was initially employed during the formation of an ancestral enamel in stem-osteichthyan; SCPP5 was subsequently replaced by AMEL in sarcopterygians, or AMEL was replaced by SCPP5 in actinopterygians. It is also conceivable, however, that AMEL arose in sarcopterygians and SCPP5 in actinopterygians. Studies of gene-disrupted mice showed that both AMBN and ENAM are necessary for enamel ribbon formation, the unique mineralization process of enamel, whereas AMEL is not (Smith et al., 2016; Liang et al., 2019). Furthermore, a thin enamel can form in AMEL-deficient mice and in toothed whales that have deleterious mutations in AMEL, if both AMBN and ENAM are functional (Gibson et al., 2001; Kawasaki et al., 2020). These results support the presence of ancestral enamel that formed enamel ribbons in a matrix containing AMBN and ENAM, but no AMEL or SCPP5. We hypothesize that both true enamel and ganoin originated from this ancestral enamel (Figures 1A and 5).

Dental acrodin arose with dental ganoin among stem-actinopterygians. It was previously hypothesized that acrodiin of teleosts is a composite of dentine and enamel (Shellis and Miles, 1974). Our observation of acrodin formation in gar confirms this hypothesis. Thus, acrodin evolution can be explained partly by early expression of ganoin matrix genes on the tooth cap during dentine formation, as previously suggested (Smith, 1992, 1995). Acrodin is similar in gar and zebrafish, but odontoblasts contribute more to acrodin formation in zebrafish by expressing scpp5 and enam.

Ancient state estimates suggest that dental enamel arose independently in stem-sarcopterygians and non-teleost actinopterygians. As we discussed above, collagen enamel is better described as collagen ganoin in gar and many fossil actinopterygians, which suggests that ganoin matrix gene expression on the tooth collar was critical to the evolution of collagen enamel. Expression of scpp5, ambn, and enam during the formation of collagen enamel in gar and collagen enameloid in zebrafish suggests an evolutionary relationship of these two tissues and reinforces the previous hypothesis that the evolution of collagen enameloid involved early expression of ganoin matrix genes during dentine formation (Smith, 1992, 1995). Consequently, collagen enamel found in various non-teleost actinopterygians (Ørvig, 1978; Schultze, 2016) was replaced by collagen enameloid in teleosts (Figure 1A). Enameloid is also found on teeth in larval urodeles (Assaraf-Weill et al., 2014; Berkovitz and Shellis, 2016), but in no other sarcopterygians (Figures S3C–S3F), indicating its independent origin in urodeles.

The results of our present study and previous studies using other methods and other species suggest that various hypermineralized tissues in modern osteichthyans can be classified genetically into true enamel, ganoin, and a diversity of enameloids; collagen enamel, acrodin, and collagen enameloid in actinopterygians are evolutionarily related to scale ganoin. A previous study supported the homology between ganoin and enamel largely based on the expression of gar ambn and enam in the skin (Qu et al., 2015); however, if acrodin and collagen enameloid arose as a composite of dentine and ganoin, neither acrodin nor collagen enameloid could be a ganoin homolog, even though AMBN and ENAM are expressed during formation. Our results show that orthologous SCPP gene expression may be insufficient to explain the differences between these tissues. The evolution of true enamel, ganoin, and enameloids can be better explained by various
changes of matrix SCPP genes, including spatiotemporal shifts in their expression. Hypermineralized tissues in osteichthyan s appear to have coevolved with their matrix SCPP genes.

Limitations of the study
In extant actinopterygians, ganoin is found only in bichirs and gars. Although scales of bichirs consist of ganoin, dentine, and bone, scales of gars lack dentine. However, ganoin formation is similar in both clades (Sire, 1995), and bichir ambn, enam, and scpp5 are presumably also expressed during scale and tooth formation in a manner similar to their gar orthologs. In teleosts, comprising ~30,000 species (Nelson et al., 2016), both acrodin and collar enameloid are found in various species. It was reported, however, that acrodin and collar enameloid of the common eel are covered with a hypermineralized layer formed by IDE cells, hence a type of enamel (Shellis and Miles, 1976). Although this layer remains to be confirmed, hypermineralized tissues may vary in some teleosts. Moreover, ambn, enam, and scpp5 are all found in zebrafish and fugu (Figure S1), whereas ambn and/or enam were secondarily lost in some teleosts (Lv et al., 2017). The lack of these genes suggests modifications of acrodin and collar enameloid, or a loss of hypermineralized tissues. Examination of hypermineralized tissues in various teleosts would elucidate the evolution and adaptation of hypermineralized tissues in diverse teleosts.

Resource availability

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Kazuhiko Kawasaki (kuk2@psu.edu).

Materials availability
The anti-gar Scpp5 antibody generated in this study is available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability
The nucleotide sequences generated during this study are available at GenBank (accession numbers: MG010658-MG010662).

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.102023.

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AUTHOR CONTRIBUTIONS
Conceptualization, K.K., P.C.J.D., and M.I.; Investigation, all authors; Writing and Supervision, K.K. and P.C.J.D.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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Supplemental Information

Coevolution of enamel, ganoin, enameloid, and their matrix SCPP genes in osteichthyans

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| Exons          | AMELX_human | AMEL_platypus | AMEL_pig | AMEL_Anolis | AMEL_caiman | AMEL_Xenopus | AMEL_lungfish | AMEL_coelacanth |
|---------------|-------------|--------------|----------|-------------|-------------|--------------|--------------|-----------------|
| exon2         | MGTWIFACLGGLAFAFAMELX            | MGTWIFACLGGLAFAFAMELX            | MGTWIFACLGGLAFAFAMELX            | MGTWIFACLGGLAFAFAMELX            | MGTWIFACLGGLAFAFAMELX            | MGTWIFACLGGLAFAFAMELX            | MGTWIFACLGGLAFAFAMELX            | MGTWIFACLGGLAFAFAMELX            |
| exon3         | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            |
| exon4         | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            |
| exon5         | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            |

| Exons          | SCPP5_medaka | SCPP5_tilapia | SCPP5_fugu | SCPP5_stickleback | SCPP5_zebrafish | SCPP5_eel | SCPP5_gar | SCPP5_bichir |
|---------------|-------------|--------------|-----------|-------------------|---------------|----------|---------|-----------|
| exon2         | SCPP5_medaka | SCPP5_tilapia | SCPP5_fugu | SCPP5_stickleback | SCPP5_zebrafish | SCPP5_eel | SCPP5_gar | SCPP5_bichir |
| exon3         | SCPP5_medaka | SCPP5_tilapia | SCPP5_fugu | SCPP5_stickleback | SCPP5_zebrafish | SCPP5_eel | SCPP5_gar | SCPP5_bichir |
| exon4         | SCPP5_medaka | SCPP5_tilapia | SCPP5_fugu | SCPP5_stickleback | SCPP5_zebrafish | SCPP5_eel | SCPP5_gar | SCPP5_bichir |
| exon5         | SCPP5_medaka | SCPP5_tilapia | SCPP5_fugu | SCPP5_stickleback | SCPP5_zebrafish | SCPP5_eel | SCPP5_gar | SCPP5_bichir |

| Exons          | AMELX_human | AMEL_platypus | AMEL_pig | AMEL_Anolis | AMEL_caiman | AMEL_Xenopus | AMEL_lungfish | AMEL_coelacanth |
|---------------|-------------|--------------|----------|-------------|-------------|--------------|--------------|-----------------|
| exon6         | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            |
| exon7         | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            |
| exon8         | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            |
| exon9         | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            | AMELX            |

YY -><- P/Q-rich core region (P/Q)
Expression of bichir scpp5 in the jaw and skin, detected by RT-PCR.
|        | exon7                                      | exon8                                      | exon9                                      | exon10                                     |
|--------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|
| AMBN human | LSVDFADGPS | LCOQSEP | LGLVDAFFGST | IYAR-LISHG-- | RPN--KSA |
| AMBN platypus | LALDETVLRES | LCOQSEP | LGLVDAFFGST | IYAR-LISHG-- | RPKA--KSG |
| AMBN caiman | LALDETVLRES |                                                                 | MIVLVNGLG | MIVG--KSA |
| AMBN Anolis | LALDETVLRES |                                                                 | MIVLVNGLG | MIVG--KSA |
| AMBN Xenopus |                                                                 |                                                                 | TIMNKLLEDAGETIQD-AAGTVNYQGM |
| AMBN lungfish |                                                                 |                                                                 | IYAR--GWAVGK-DYKMK--IYAR |
| AMBN coelacanth |                                                                 |                                                                 | VSXVGNAYT--PHTIPTIPTIPTI-- |
| AMBN bichir |                                                                 |                                                                 | VSXVGNAYT--PHTIPTIPTIPTI-- |
| AMBN sturgeon |                                                                 |                                                                 | VSXVGNAYT--PHTIPTIPTIPTI-- |
| AMBN gar |                                                                 |                                                                 | VSXVGNAYT--PHTIPTIPTIPTI-- |
| AMBN zebrafish |                                                                 |                                                                 | VSXVGNAYT--PHTIPTIPTIPTI-- |
| AMBN medaka |                                                                 |                                                                 | VSXVGNAYT--PHTIPTIPTIPTI-- |
| AMBN tilapia |                                                                 |                                                                 | VSXVGNAYT--PHTIPTIPTIPTI-- |
| AMBN fugu |                                                                 |                                                                 | VSXVGNAYT--PHTIPTIPTIPTI-- |

|        | exon11                                      | exon12                                      |
|--------|-------------------------------------------|-------------------------------------------|
| AMBN human | L--YPGMLYVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN platypus | L--YPELVYVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN caiman | L--HEPLYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN Anolis | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN Xenopus | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN lungfish | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN coelacanth | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN bichir | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN sturgeon | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN gar | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN zebrafish | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN medaka | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN tilapia | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |
| AMBN fugu | L--YPELYVVP--------GANQL                     | N--APARLGIMSSEEVA |

|        | exon12                                      |
|--------|-------------------------------------------|
| AMBN human | N--APARLGIMSSEEVA |
| AMBN platypus | N--APARLGIMSSEEVA |
| AMBN caiman | N--APARLGIMSSEEVA |
| AMBN Anolis | N--APARLGIMSSEEVA |
| AMBN Xenopus | N--APARLGIMSSEEVA |
| AMBN lungfish | N--APARLGIMSSEEVA |
| AMBN coelacanth | N--APARLGIMSSEEVA |
| AMBN bichir | N--APARLGIMSSEEVA |
| AMBN sturgeon | N--APARLGIMSSEEVA |
| AMBN gar | N--APARLGIMSSEEVA |
| AMBN zebrafish | N--APARLGIMSSEEVA |
| AMBN medaka | N--APARLGIMSSEEVA |
| AMBN tilapia | N--APARLGIMSSEEVA |
| AMBN fugu | N--APARLGIMSSEEVA |

|        | exon12                                      |
|--------|-------------------------------------------|
| AMBN human | N--APARLGIMSSEEVA |
| AMBN platypus | N--APARLGIMSSEEVA |
| AMBN caiman | N--APARLGIMSSEEVA |
| AMBN Anolis | N--APARLGIMSSEEVA |
| AMBN Xenopus | N--APARLGIMSSEEVA |
| AMBN lungfish | N--APARLGIMSSEEVA |
| AMBN coelacanth | N--APARLGIMSSEEVA |
| AMBN bichir | N--APARLGIMSSEEVA |
| AMBN sturgeon | N--APARLGIMSSEEVA |
| AMBN gar | N--APARLGIMSSEEVA |
| AMBN zebrafish | N--APARLGIMSSEEVA |
| AMBN medaka | N--APARLGIMSSEEVA |
| AMBN tilapia | N--APARLGIMSSEEVA |
| AMBN fugu | N--APARLGIMSSEEVA |
| Species          | AMBN | Exon 13                     |
|------------------|------|-----------------------------|
| **AMBN_human**   |      | GGGRE- |---------------------------------|----------------|-----------------|
|                  |      | DMAYGAMTF | GGGRE-DPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_platypus** |      | GGGG- |---------------------------------|----------------|-----------------|
|                  |      | EPLAYGIF | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_caiman**  |      | GGGG- |---------------------------------|----------------|-----------------|
|                  |      | EVPAYGAMTF | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_Anolis**  |      | GGGG- |---------------------------------|----------------|-----------------|
|                  |      | GAPAYRAPG | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_Xenopus** |      | GGRTGAAHAF | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_lungfish** |      | GFPIQF |---------------------------------|----------------|-----------------|
|                  |      | YALGHQNLPQNVQGAISSEEIQANRAAGAAAAV | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_coelacanth** |      | AAGG- |---------------------------------|----------------|-----------------|
|                  |      | VA | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_gar**     |      | AAGG- |---------------------------------|----------------|-----------------|
|                  |      | VA | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_zebrafish** |      | NMGR-VNMHLPAIRGNVFPVSGPQTVVPG | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_medaka**  |      | MQAK-LNQMSV | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_tilapia** |      | MAAT-MGQLGV | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_fugu**    |      | AELLPLTQTRSV | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_zebrafish** |      | MGPTIPEVIPTGQGSPL- | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_medaka**  |      | SLPTNRNLVRSKA | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_tilapia** |      | LQPMQETLV | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |
| **AMBN_fugu**    |      | AELLPLTQTRSV | GGGRDPMAYGAMTF | VAAKGP | EKEGAG- | GSDR | LEAN | BNLESENFL | TLTELA | FAHAG- | LLV |

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**Note:** The above table continues with the sequences for other species, but due to the nature of the table, the full content is not displayed in this representation.
ENAM human

exon9 (continued)

ENAM_zebrafish

ENAM_gar

ENAM_fugu

ENAM_coelacanth

ENAM_bichir

ENAM_platypus

ENAM_gar

ENAM_zebrafish

ENAM_anacon

ENAM_platypus

ENAM_fugu

ENAM_coelacanth

ENAM_bichir

ENAM_platypus

ENAM_fugu

ENAM_coelacanth

ENAM_bichir

ENAM_platypus

ENAM_fugu

ENAM_coelacanth

ENAM_bichir

ENAM_platypus

ENAM_fugu

ENAM_coelacanth

ENAM_bichir

ENAM_platypus

ENAM_fugu

ENAM_coelacanth

ENAM_bichir

ENAM_platypus

ENAM_fugu

ENAM_coelacanth

ENAM_bichir

ENAM_platypus

ENAM_fugu

ENAM_coelacanth

ENAM_bichir

ENAM_platypus

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ENAM_coelacanth

ENAM_bichir

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ENAM_coelacanth

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ENAM_coelacanth

ENAM_bichir

ENAM_platypus

ENAM_fugu

ENAM_coelacanth

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Figure S1. Multiply aligned amino acid sequences encoded by matrix SCPP genes, Related to Figure 1.

(A) Multiply aligned amino acid sequences encoded by AMEL and SCPP5, their chromosomal locations, and expression of bichir scpp5 in the jaw and skin. Aromatic residues, negatively charged residues, and positively charged residues are shown in sky blue, blue, and brown, respectively, while Pro, Gln, and potentially phosphorylated Ser residues (potential pSer residues: Ser residues in Ser-Xaa-Glu or Ser-Xaa-pSer sequences, where Xaa represents any amino acids, are known to be phosphorylated in SCPPs.) (Kawasaki and Amemiya, 2014) are highlighted in yellow, blue, and green, respectively. Uninterrupted Pro-Xaa-Yaa (Yaa also represents any amino acid; PXY) repeats are shown with dashed underlines. The three conserved modules, the aromatic residue-rich region (YY), the Pro/Gln-rich region containing uninterrupted PXY repeats (P/Q), and the hydrophilic C-terminus (DE) are shown at the bottom. Unaligned sequence gaps are shown by dashes. Amino acid sequences were retrieved from GenBank (accession numbers for AMEL: NP_873632.1 for humans, NP_998965.1 for pig, reconstructed from GCA_000002275 for platypus, XP_003228746.1 for Anolis, AAC78133.1 for caiman, AAZ23149.1 for axolotl, NP_001107153.1 for Xenopus, AU58914.1 for lungfish, and reconstructed from GCA_000225785.1 for coelacanth; accession numbers for SCPP5: AU58917.1 for bichir, XP_015200896.1 for gar, XP_035273951.1 for eel, NP_001138708.1 for zebrafish, reconstructed from GCA_000180675.1 for stickleback, NP_001032946.1 for fugu, reconstructed from GCA_000188235.2 for tilapia, and reconstructed from GCA_002234675.1 for medaka). Note that all teleost species used for multiple amino acid sequence alignments (A, B, and C) belong to different orders. In sarcopterygians, AMEL is located within intron 1 of ARHGAP6, while in actinopterygians SCPP5 is located within a P/Q-rich SCPP gene cluster (Kawasaki et al., 2017). In L. chalumnae, locations of genes are shown as contig names (JHxxxxxx). Expression of bichir scpp5 was detected in the jaw and skin that secretes the ganoin matrix on scales, but not in the skeletal muscle (negative control). Bichir gapdh (glyceraldehyde 3-phosphate dehydrogenase) was used as the positive control.

(B) Multiply aligned amino acid sequences encoded by AMBN. Cys residues is shown in red. See the legend above. AMBN in sturgeon, medaka, and tilapia has a small last exon, and the C-terminus is shown by a period. The amino acid sequences shown under exon 6 of human AMBN are encoded by two different exons in bichir, sturgeon, and gar (separated by an underscore). In a previous study, ambn was named scpp6 in teleosts (Kawasaki, 2009). However, conservation of the amino acid sequences encoded by exons 11 and 12 (corresponds to exon 12 of human AMBN) between AMBN and SCPP6, the absence of the entirely untranslated last exon in both ANBN and SCPP6, the presence and locations of four potential pSer residues, and an equivalent chromosomal location of gar ambn and zebrafish scpp6 (between scpp3 genes and scpp7) implies the orthology of AMBN and SCPP6 (Qu et al., 2015; Braasch et al., 2016; Kawasaki et al., 2017). In the present study, the orthology of AMBN and SCPP6 was confirmed by a similar expression pattern during tooth development. Amino acid sequences were retrieved from GenBank (AAG35772.1 for humans, XP_007667389.2 for platypus,
AAK92227.1 for caiman, XP_016846623.1 for Anolis, XP_002938667.2 for Xenopus, AYU58913.1 for lungfish, XP_006011890.1 for coelacanth, AYU58915.1 for bichir, reconstructed from SRX424534 for sturgeon, AMD08894.1 for gar, NP_001138709.1 for zebrafish, reconstructed from GCA_002234675.1 for medaka, reconstructed from GCA_000188235.2 for tilapia, and NP_001032945.1 for fugu).

(C) Multiply aligned amino acid sequences encoded by exons 3, 4, 5, 6, 7, 8, and a 5' half of exon 9 of ENAM. The amino acid sequence of porcine 32-kDa enamelin (Tanabe et al., 1990; Yamakoshi et al., 1998) was underlined. Small amino acids (Ala and Gly) in the 32-kDa enamelin and corresponding regions are highlighted in grey. In the region corresponding to exon 6 of human ENAM, many small duplicate exons were identified in gar and bichir enam genes but are not shown in the figure. Amino acid sequence similarities in the portion located C-terminal to the 32-kDa enamelin (not highlighted or not shown in color) are low or undetectable between phylogenetically distant vertebrates (e.g., tetrapods and teleosts). In teleosts, enam was originally referred to as fa93e10 (Goldsmith et al., 2003). However, sequence similarities in the 32-kDa enamelin and its N-terminal region encoded by ENAM and FA93E10, the absence of the entirely untranslated last exon in both ENAM and FA93E10, the presence and locations of four pSer residues, and an equivalent chromosomal location of far enam and zebrafish enam (immediately downstream of scpp5) imply the orthology of ENAM and FA93E10 (Qu et al., 2015; Braasch et al., 2016; Kawasaki et al., 2017). In the present study, the orthology of ENAM and FA93E10 was confirmed by a similar expression pattern during tooth development. Amino acid sequences were retrieved from GenBank (AAG43242.1 for humans, NP_999406.1 for pig, reconstructed from GCA_000002275.2 for platypus, ADJ67842.1 for Anolis, NP_001139215.1 for Xenopus, reconstructed from GCA_000225785.1 for coelacanth, AYU58916.1 for bichir, AMD08897.1 for gar, NP_001139028.1 for zebrafish, CDQ64219.1 for trout, reconstructed from GCA_000180735.1 for fugu, reconstructed from GCA_002234675.1 for medaka, and XP_003454248.1 for tilapia).
A

ISH: gar teeth

ISH: zebrafish teeth
Figure S2. Expression of gar and zebrafish scpp5, ambn, and enam genes in teeth and/or scales, and distribution of gar Scpp5 in teeth and scales, Related to Figures 2-4.
(A) ISH analysis of gar teeth, gar scales, and zebrafish teeth. Expression of scpp5, ambn, and enam genes were detected using antisense (-) probes, but not using sense (+) probes, which serve as negative controls. Images of HE staining are shown for gar scales and zebrafish teeth. All our ISH results are summarized in Figure 2P. Abbreviations: acr, acrodin; bo, bone; ga, ganoin; ide, inner dental epithelial cells; ige, inner ganoin epithelial cells; od, odontoblasts. Scale bar, 50 µm.

(B) Original images for optical IHC analysis (Figures 3A-3F) without enhancing image contrast. See the legend of Figure 3. Scale bar, 100µm (upper row) or 20 µm (lower row).

(C) TEM IHC analysis of gar Scpp5 in acrodin, collar enamel, and ganoin. PBS was used as negative controls. Abbreviations: acr, acrodin, cem, collar enamel; de, dentine; ga, ganoin; ide, inner dental epithelial cells; ige, inner ganoin epithelial cells. Scale bar, 500 nm.
Dental Enamel (unconstrained tree)

- no Enamel on teeth
- Enamel on teeth
Dermal Ganoine (constrained tree)
Dermal Ganoin (unconstrained tree)
Dermal hypermineralized tissue (constrained tree)
Figure S3. Reconstructing ancestral states of hypermineralized tissues, Related to Figure 5.

(A) Constrained tree used in ancestral state estimation. The GAP clade is constrained to a stem-osteichthyan affinity. Reliabilities of branches are shown at nodes as posterior probabilities.

(B) Unconstrained tree used in ancestral state estimation. The affinity of the GAP clade was unconstrained in the analysis and is resolved to a stem-sarcopterygian affinity. Reliabilities of branches are shown at nodes as posterior probabilities.

(C) Prediction of ancestral states of dental acrodin - GAP clade stem-osteichthyans, shown as pie charts.

(D) Prediction of ancestral states of dental acrodin - GAP clade unconstrained, shown as pie charts.

(E) Prediction of ancestral states of dental enameloid - GAP clade stem-osteichthyans, shown as pie charts.

(F) Prediction of ancestral states of dental enameloid - GAP clade unconstrained, shown as pie charts.

(G) Prediction of ancestral states of dermal enameloid - GAP clade stem-osteichthyans, shown as pie charts.

(H) Prediction of ancestral states of dermal enameloid - GAP clade unconstrained, shown as pie charts.

(I) Prediction of ancestral states of dental enamel - GAP clade stem-osteichthyans, shown as pie charts.

(J) Prediction of ancestral states of dental enamel - GAP clade unconstrained, shown as pie charts.

(K) Prediction of ancestral states of dermal enamel - GAP clade stem-osteichthyans, shown as pie charts.

(L) Prediction of ancestral states of dermal enamel - GAP clade unconstrained, shown as pie charts.

(M) Prediction of ancestral states of dermal ganoin - GAP clade stem-osteichthyans, shown as pie charts.

(N) Prediction of ancestral states of dermal ganoin - GAP clade unconstrained, shown as pie charts.

(O) Prediction of ancestral states of dermal enamel+ganoin - GAP clade stem-osteichthyans, shown as pie charts.
(P) Prediction of ancestral states of dermal enamel or ganoin - GAP clade unconstrained, shown as pie charts.
(Q) Prediction of ancestral states of dermal enamel, ganoin, or enameloid - GAP clade stem-osteichthyans, shown as pie charts.
(R) Prediction of ancestral states of dermal enamel, ganoin, or enameloid - GAP clade unconstrained, shown as pie charts.
(S) Cladoselache skin denticles [specimen P.9294 (NHMUK)]. Detail of boxed areas (1-4) in overview is enlarged. The dentin tubules (arrowheads), protruding through the enameloid layer (arrow), are clearly visible.
Table S1. Expression levels of SCPP genes in the skin, Related to Figure 2. Relative expression levels of SCPP genes in the skin were estimated as fragments per kilobase of transcript per million mapped reads (FPKM values) (Trapnell et al., 2010). \(^1\)Coverage shows the estimate for the absolute depth of read coverage across the whole transcript. \(^2\)Conf represents the 95% confidence interval.
| Taxa               | Enamel | Ganoin | Enamel | Enamel+Ganoin | Enamel+Ganoin+Enameloid | Accrod | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enamel | Enema...
| Genus                      | 0/1 | 0  | 0  | 0  | 0/1 | -  | -  | -  |
|----------------------------|-----|----|----|----|-----|----|----|----|
| Kathemacanthus             |     |    |    |    |     |    |    |    |
| Kenichthyx                 |     |    |    |    |     |    |    |    |
| Kentuckia                  |     |    |    |    |     |    |    |    |
| Kujdanowiaspis             |     |    |    |    |     |    |    |    |
| Latiascanthus              |     |    |    |    |     |    |    |    |
| Ligulalepis                |     |    |    |    |     |    |    |    |
| Lophositeus                |     |    |    |    |     |    |    |    |
| Lunaspis                   |     |    |    |    |     |    |    |    |
| Lupopsyrus                 |     |    |    |    |     |    |    |    |
| Macropetalichthys          |     |    |    |    |     |    |    |    |
| Materpisicus               |     |    |    |    |     |    |    |    |
| Meemanniaglæcki            |     |    |    |    |     |    |    |    |
| Mesacanthus                |     |    |    |    |     |    |    |    |
| Microbrachius              |     |    |    |    |     |    |    |    |
| Miguashaia                 | 0/1 |    |    |    | 0/1 |    |    |    |
| Lepisosteus                | 0   | 1  | 0  | 1  | 1   | 1  | 0  | 1  |
| Polypterus                 | 0   | 0  | 0  | 1  | 1   | 1  | 1  | 1  |
| Mimipscis                  | 0   | 0  | 0  | 1  | 1   | 1  | 1  | 1  |
| Myothyomassa               | 0   | 0  | 0  | 1  | 1   | 1  | 1  | 1  |
| Obtusacanthus              | 0/1 | 0  | 0  | 0  | 0/1 | -  | -  | -  |
| Onychodus                  | 0   | 0  | 0  | 0  | 0   | 0  | 0  | 0  |
| Onychoselache              |     |    |    |    |     |    |    |    |
| Orthacanthus               |     |    |    |    |     |    |    |    |
| Osorioichthys              |     |    |    |    |     |    |    |    |
| Osteolepis                 |     |    |    |    |     |    |    |    |
| Parabuchanosteus           |     |    |    |    |     |    |    |    |
| Parayunnanolepis           |     |    |    |    |     |    |    |    |
| Paraxius                   | 0/1 | 0  | 0  | 0  | 0/1 | 0  | 0  | 0  |
| Poracanthodies             | 1   | 0  | 0  | 0  | 0   | 1  | 0  | 1  |
| Porolepis                  | 0   | 0  | 1  | 1  | 1   | 1  | 1  | 1  |
| Powichthyx                 | 0   | 0  | 1  | 1  | 1   | 0  | 0  | 1  |
| Promesacanthus             | 0/1 | 0  | 0  | 0  | 0/1 | -  | -  | -  |
| Psalepis                   |     |    |    |    |     |    |    |    |
| Pterichthydoides           |     |    |    |    |     |    |    |    |
| Plomacanthus               | 0/1 | 0  | 0  | 0  | 0/1 | 0  | 1  | 0  |
| Pucapampella               | 0/1 | 0  | 0  | 0  | 0/1 | 0  | 1  | 0  |
| Quasipetalichthys          | 0   | 0  | 0  | 0  | 0   | 0  | 1  | 0  |
| Ramiriusceraeza            | 0/1 | 0  | 0  | 0  | 0/1 | 0  | 1  | 0  |
| Remigolepis                | 0   | 0  | 0  | 0  | 0   | 0  | 0  | 0  |
| Rhadinacanthus             | 1   | 0  | 0  | 0  | 0   | -  | -  | -  |
| Rhamphodopsis              | 0/1 | 0  | 0  | 0  | 0/1 | 0  | 0  | 0  |
| Romundinia                 | 1   | 0  | 0  | 0  | 0   | 1  | 0  | 1  |
| Sigaspi                    | 0/1 | 0  | 0  | 0  | 0/1 | 0  | 1  | 0  |
| Sinolespis                 | 0/1 | 0  | 0  | 0  | 0/1 | 0  | 1  | 0  |
| Styloichthys               |     |    |    |    |     |    |    |    |
| Latimeria                  |     |    |    |    |     |    |    |    |
| Taniothys                  |     |    |    |    |     |    |    |    |
| Tetanopsyrus               |     |    |    |    |     |    |    |    |
| Tristichius                |     |    |    |    |     |    |    |    |
| Vemicomacanthus            |     |    |    |    |     |    |    |    |
| Wuttigoonaspis             |     |    |    |    |     |    |    |    |
| Youngolepis                |     |    |    |    |     |    |    |    |
| Yunmolepis                 |     |    |    |    |     |    |    |    |
| Andreolepis                |     |    |    |    |     |    |    |    |

| Table S2. Presence or absence of enameloid, ganoin, enamel, enamel or ganoin, enamel, ganoin, or enameloid in the dermal skeleton, and acrodin, enameloid, and enamel on teeth, Related to Figure 5. 0, 1, and 0/1 represent the absence, presence, and unknown status of the tissue, respectively. “-“ in dental characters represents the absence of teeth. |
| Taxa                  | Minimum age (Mya) | Reference          |
|----------------------|-------------------|--------------------|
| Galeaspida           | 432.6             |                    |
| Osteostraci          | 437.4             |                    |
| Acanthodes           | 298               | King et al. 2016   |
| Achoanis             | 412               | King et al. 2016   |
| Akmonistion          | 327               | King et al. 2016   |
| Austroptyctodus       | 383               | King et al. 2016   |
| Bothriolepis         | 383               | King et al. 2016   |
| Brachyacanthus        | 415               | King et al. 2016   |
| Brindabellaspis       | 401               | King et al. 2016   |
| Brochoadmones         | 415               | King et al. 2016   |
| Buchananosteus       | 408               | King et al. 2016   |
| Campbellodus          | 383               | King et al. 2016   |
| Cassidiceps          | 415               | King et al. 2016   |
| Cheiracanthus         | 388               | King et al. 2016   |
| Cheirolepis           | 388               | King et al. 2016   |
| Chondrenchelys       | 338               | King et al. 2016   |
| Cladodoides           | 375               | King et al. 2016   |
| Cladoselache          | 360               | King et al. 2016   |
| Climatius             | 415               | King et al. 2016   |
| Cobelodus             | 325               | King et al. 2016   |
| Coccosteus            | 388               | King et al. 2016   |
| Compagopiscis         | 383               | King et al. 2016   |
| Cowratepis            | 383               | King et al. 2016   |
| Cuilmacanthis         | 385               | King et al. 2016   |
| Debeverius            | 320               | King et al. 2016   |
| Diabolepis            | 412               | King et al. 2016   |
| Dialipina             | 401               | King et al. 2016   |
| Diandongpetalichthys  | 417               | King et al. 2016   |
| Dicksonosteus         | 411               | King et al. 2016   |
| Diplacanthus          | 388               | King et al. 2016   |
| Doliodus              | 395               | King et al. 2016   |
| Eastmanosteus         | 383               | King et al. 2016   |
| Entelognathus         | 424               | King et al. 2016   |
| Eurycaraspis          | 385               | King et al. 2016   |
| Eusthenopteron        | 380               | King et al. 2016   |
| Euthacanthus          | 415               | King et al. 2016   |
| Gavinaspis            | 412               | King et al. 2016   |
| Gemuendina            | 408               | King et al. 2016   |
| Gladiobranchus        | 415               | King et al. 2016   |
| Glyptolepis           | 388               | King et al. 2016   |
| Gogonasus             | 383               | King et al. 2016   |
| Groenlandaspis        | 385               | King et al. 2016   |
| Guiyu                 | 424               | King et al. 2016   |
| Gyracanthides         | 359.3             | Warren et al. 2000 |
| Scylophirhurus        | 0                 |                    |
| Hamiltonichthys       | 302               | King et al. 2016   |
| Helodus               | 311               | King et al. 2016   |
| Holonema              | 383               | King et al. 2016   |
| Homalacanthis         | 380               | King et al. 2016   |
| Howqualepis           | 385               | King et al. 2016   |
| Incisoscutum          | 383               | King et al. 2016   |
| Ischnacanthis         | 415               | King et al. 2016   |
| Jagonna               | 375               | King et al. 2016   |
| Jansusicus            | 415               | King et al. 2016   |
| Kathemacanthis        | 415               | King et al. 2016   |
| Kenichthys            | 396               | King et al. 2016   |
| Kentuckia             | 347               | King et al. 2016   |
| Kudjanowraspis        | 411               | King et al. 2016   |
| Latviscirothrus       | 404               | King et al. 2016   |
| Ligualepis            | 401               | King et al. 2016   |
| Lophoesteus           | 416               | Cunningham et al. 2012 |
| Lunaspis              | 408               | King et al. 2016   |
| Lupopsyrus            | 415               | King et al. 2016   |
| Macropetalichthys     | 390               | King et al. 2016   |
| Materpiscis           | 383               | King et al. 2016   |
| Meemannia             | 412               | King et al. 2016   |
| Mesacanthis           | 415               | King et al. 2016   |
| Microbrachiensis      | 386               | King et al. 2016   |
| Miguashaia            | 380               | King et al. 2016   |
| Lepisosteus           | 0                 |                    |
| Polypterus            | 0                 |                    |
| Mimipiscis            | 383               | King et al. 2016   |
| Moythomasia           | 383               | King et al. 2016   |
| Taxon                  | Age  | Reference          |
|-----------------------|------|--------------------|
| Obitusacanthus        | 415  | King et al. 2016   |
| Onychodus             | 383  | King et al. 2016   |
| Onychoselachus        | 336  | King et al. 2016   |
| Orthacanthus          | 290  | King et al. 2016   |
| Osorioichthys         | 367  | King et al. 2016   |
| Osteolepis            | 388  | King et al. 2016   |
| Parabuchanosteus      | 401  | King et al. 2016   |
| Parayunnanolepis      | 412  | King et al. 2016   |
| Parexus               | 415  | King et al. 2016   |
| Poracanthodes         | 417  | King et al. 2016   |
| Porolepis             | 411  | King et al. 2016   |
| Powichthys            | 411  | King et al. 2016   |
| Promesacanthus        | 415  | King et al. 2016   |
| Psorolepis            | 416  | King et al. 2016   |
| Andreolepis           | 424  | Chen et al. 2016   |
| Plerichthyodes        | 389  | King et al. 2016   |
| Plomacanthus          | 415  | King et al. 2016   |
| Pucapampella          | 388  | King et al. 2016   |
| Quasipetalichthys     | 385  | King et al. 2016   |
| Ramirosuarezia        | 392  | King et al. 2016   |
| Remigolepis           | 366  | King et al. 2016   |
| Rhadinacanthus        | 386.9| Lukševičs et al. 2010 |
| Rhamphodopis          | 388  | King et al. 2016   |
| Romundina             | 415  | King et al. 2016   |
| Sigaspis              | 412  | King et al. 2016   |
| Sinolespis            | 358.5| Zhu et al. 2000    |
| Styloichthys          | 412  | King et al. 2016   |
| Latimeria             | 0    |                    |
| Tamiobatis            | 360  | King et al. 2016   |
| Tetanopsyrus          | 415  | King et al. 2016   |
| Tristychius           | 336  | King et al. 2016   |
| Vernicomacanthus      | 415  | King et al. 2016   |
| Wuttagoonaspis        | 393  | King et al. 2016   |
| Youngolepis           | 412  | King et al. 2016   |
| Yunnanolepis          | 415  | King et al. 2016   |

Table S3. Tip ages used for the ancestral state estimation, Related to Figure 5.
Transparent Methods

Bioinformatic analyses

We searched for SCPP genes in RNA-seq datasets (GenBank accession numbers: SRX796494 and SRX1016233-SRX1016241 for lungfish, SRX796491 and SRX1386644-SRX1386647 for bichir, and SRX424533 and SRX424534 for sturgeon) by tblstn (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990) using amino acid sequences of gar orthologs as queries (Kawasaki et al., 2017).

Relative expression levels of gar SCPP genes were estimated as FPKM values (Trapnell et al., 2010), calculated for a dataset of gar skin (GenBank accession number, SRP042013) (Braasch et al., 2016) using Galaxy (https://usegalaxy.org/) (Afgan et al., 2016). The dataset was retrieved from the EBI SRA database, trimmed using Trimmatic (Bolger et al., 2014), aligned with the gar genome sequence (LepOcu1) using TopHat (Kim et al., 2013). FPKM values and confidence intervals were calculated using Cufflinks (Trapnell et al., 2010) based on BAM files obtained by the TopHat analysis. All default conditions were used in Galaxy analysis, except additional options in Trimmomatic (ILLUMINAACLIP and MINLEN=50) and Cufflinks (multi-read correction). Genomic coordinates of SCPP genes were determined using Splign (http://www.ncbi.nlm.gov/) (Kapustin et al., 2008), and used for the Cufflinks analysis.

Molecular analysis

All animals used in our study (gar, 16-55 cm in total length; zebrafish, 3.0 cm in total length; bichir, 20 cm in total length; and lungfish, 20 cm in total length; sex not determined for these animals) were sacrificed according to the guidelines issued by the Ministry of Justice in Japan. SCPP genes identified in bichir and lungfish RNA-seq datasets were confirmed by PCR using cDNA libraries made from a tooth plate (lungfish) and tooth germs (bichirs), as described (Braasch et al., 2016). These libraries were made using SMART cDNA library construction kit (Takara Bio), and PCR products were size fractionated by agarose gel electrophoresis, purified from the agarose gel using the FastGene Gel/PCR Extraction Kit (Nippon Genetics), and ligated into T-vector pMD20 (Takara Bio). The ligation mix was then used to transform E.
coli HST08 Premium Competent Cells (Takara Bio). Exon-intron borders were also determined by PCR using genomic DNA as the substrate. For expression analysis of bichir scpp5, total RNA molecules were isolated using RNAiso Plus (Takara Bio) and cDNA was synthesized using the PrimerScript II 1st strand cDNA synthesis kit (Takara bio). Primer sequences used for RT-PCR are as follows: 5'-GAGACTTGCGATCTTCTTCTCTG-3' and 5'-GTGAATTGACCTGAGGCAGGA-3' for scpp5; and 5'-CACAGTTTGCCAGATGGTCC-3' and 5'-CACCACCAATTGCTTGCTC-3' for gapdh.

**ISH analysis**

For ISH analysis, jaws and skin of gar and zebrafish were fixed with neutralized 4% paraformaldehyde, decalcified with Morse's solution (10% w/v sodium citrate and 22.5% v/v formic acid), dehydrated through a graded ethanol series and xylene, embedded in paraffin, sectioned in the coronal plane at 4 µm in thickness, and mounted on glass slides. These glass slides were deparaffined, treated with Proteinase K (20 mg/ml) for 10 min, and used for hybridization (50% formamide, 10 mM Tris pH 7.6, 1xDenhart’s solution, 5% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, and 100µg/ml Escherichia coli tRNA) at 70°C (Nakatomi et al., 2006). A specific portion of scpp5, ambn, and enam were amplified from the cDNA libraries (primer sequences: 5'-GTTGGTGCTACAGCAGGAAGT-3' and 5'-GTTGTTGCTTCCCTGAACTG-3' for gar ambn; 5'-AAGGCCTCAGCTTCTTCCAGG-3' and 5'-ACTCCTTCTGATTGCTTCTG-3' for gar enam; 5'-TATTCTGAGAGCTCAAAC-3' and 5'-ATTCTGAATGGCTGGACG-3' for gar scpp5; 5'-ACTTCATCAACAGGTGCCAATC-3' and 5'-TGAAGCTCCGTTGACCTGAATCT-3' for zebrafish ambn; and 5'-CTGCCCGCTGACAGTGTTGCTGT-3' and 5'-TACAGGCCCCAACACAGTGATTG-3' for zebrafish enam), cloned using the pGEM-T Easy Vector (Promega), and labeled with digoxigenin using the DIG RNA Labeling Kit (Roche), and used for hybridization. After hybridization, glass slides were washed with 2xSSC. Hybridization signals were detected using Anti-Digoxigenin-AP, Fab fragments (Roche) and NBT/BCIP Solution (Roche), as specified.
IHC analysis

For IHC analysis, jaws and scales were fixed (4% paraformaldehyde-0.2% glutaraldehyde, 0.05M HEPES buffer pH7.4), dehydrated, and embedded in LR-White resin (LR-W, London Resin), and processed for the Protein A-gold (PAG) method (Sasagawa et al., 2012). For light microscopic analysis, semi-thin sections were made from LR-W resin block, mounted on glass slides, masked with 5% goat serum in phosphate buffered saline (PBS), and reacted with the antibody diluted 1:100 or 1:200 in PBS containing 0.5% bovine serum albumin (PBS-BSA) at 4C. These sections were washed with PBS, incubated with the PAG conjugate (gold nanoparticles 5nm, BBI) diluted 1:100 with PBS-BSA, washed with PBS, treated with the silver enhancer solution (50 mM citrate buffer, 0.85% hydroquinone, 0.1% maleic acid, 0.11% silver lactate, and 1% acacia powder) (Uchida et al., 1991) for 10 min in a dark box, and rinsed with water. These sections were then immersed in photographic fixatives, and stained with fast red or toluidine blue (Sasagawa et al., 2012).

For TEM analysis, ultrathin sections obtained from LR-W resin block were mounted on nickel grids, floated on a drop of 1% goat serum and then the antibody diluted 1:200-1:400 in PBS-BSA. These sections were subsequently washed with PBS-BSA, treated with 1% goat serum and then with the PAG conjugate diluted 1:10 in PBS-BSA. These sections were stained with platinum blue and lead citrate (additionally stained with phosphotungstic acid for some samples), and examined using TEM (JEM-1010, JEOL) (Sasagawa et al., 2016). In some sections, immunoreactions were enhanced using the silver enhancer solution (Uchida et al., 1991) or the Silver Enhancer Kit (Kirkegaard & Perry Laboratories). Polyclonal antibodies to gar Scpp5 were raised against YRQQPQQQN (SIGMA-ALDRICH).

Phylogenetic analysis

We augmented an existing phylogenetic dataset (Qiao et al., 2016) with codings for Scyliorhinus, Lepisosteus, Polypterus, and Latimeria, representing the three major living clades of jawed vertebrates. We also coded Andreolepis, a putative stem-osteichthyan (Qu et al., 2015), and coded Ligulalepis as
present for dental acrodin (Schultze, 2016; Schultze, 2018). Morphological data were analyzed using the Mk+v+G model in MrBayes 3.2.7 (Ronquist et al., 2012). We constrained the positions of the extant taxa to mitigate long branch effects: *Scyliorhinus* with *Helodus, Chondrenchelys, Debeerius, Tristychius, Hamiltonichthys, Onychoselache* (Qiao et al., 2016); *Latimeria* with *Miguashaia* (Arratia and Schultze, 2015); and *Polypterus, Lepisosteus, Kentuckia* with *Moythomasia, Mimipiscis* (Giles et al., 2017). We conducted two phylogenetic analyses of this dataset, one in which the GAP clade were constrained to be stem-osteichthyans (King et al., 2017; Lu et al., 2017), and the other in which these taxa were topologically unconstrained, yielding a stem-sarcopterygian affinity (Lu et al., 2016; Qiao et al., 2016; Choo et al., 2017). Each analysis used four independent runs of four chains over 10,000,000 generations, sampling every 10,000 generations, with 25% of burnin. Convergence was assessed using Tracer 1.7 (Rambaut et al., 2018). Results were summarized using majority rule consensus trees (Figures S3A and S3B).

**Ancestral state estimation**

We compiled data on the distribution of five dermal (enameloid, ganoin, enamel, ganoin/enamel, hypermineralized tissue) and three dental (acrodin, enameloid, enamel) skeletal characters (Table S2); we confirmed the presence of dental enameloid in *Cladoselache* (Figure S3S).

Branch lengths were estimated using the function `timePaleoPhy` in the R package paleotree (Bapst, 2012) using the ‘equal’ method (Brusatte et al., 2008), with the root age increased by 5 million years. Tip ages are from King *et al.* (King *et al.*, 2017) (Table S3) but the root (Tinn and Märss, 2018), and the crown clades of chondrichthyanas, sarcopterygians, actinopterygians, osteichthyans, and gnathostomes (Benton et al., 2015) were calibrated to be minimally as old as oldest fossil representative. Using these time-scaled topologies, we estimated ancestral states using Stochastic Character Mapping (Huelsenbeck et al., 2003) in the R package phytools (Revell, 2012). Each character was coded as a binary presence or absence state for all taxa; uncertain tip states were assigned an equal prior probability of 0.5. Taxa were excluded from an analysis when the character was
inapplicable. By running all separate analyses for each character, there is zero modelled co-variance between each character. Stochastic character mapping was run using the `make.simmap` function (Bollback, 2006) in `phytools` but with a single Q matrix for all simulations under the ‘all rates different’ and a naïve equal prior probability for absence or presence at the root. Each character model ran for 1000 simulations and each iteration used the same ‘empirical’ Q matrix with the highest likelihood transition probabilities. Results were summarized using the `describe.simmap` function and the posterior probabilities of node and tip states were plotting on each tree.
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