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New insights into the enameloid microstructure of batoid fishes (Chondrichthyes)

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Chondrichthyan teeth are capped with a hypermineralized tissue known as enameloid. Its microstructure displays a hierarchical organization that has increased in structural complexity from a homogenous single-crystallite enameloid (SCE) in early Chondricthyans to the complex multilayered enameloid found in modern sharks (consisting of bundles of crystallites arranged in intriguing patterns). Recent analyses of the enameloid microstructure in batoid fishes, focused on Myliobatiformes and fossil taxa, point to the presence of a bundled (or fibred) multilayered enameloid, a condition proposed as plesiomorphic for Batoidea. In this work, we provide further enameloid analysis for a selection of taxa covering the phylogeny of batoids. Our SEM analysis shows a superficial layer of SCE, where individualized crystallites are clearly discernable, capping the teeth in most of the species studied. A bundled double-layered enameloid was found only in a Rhinoidei, *Rhina ancylostoma* Bloch & Schneider, 1801. We conclude that the most widespread condition among extant batoids is a monolayer SCE lacking microstructural differentiation, probably plesiomorphic at least for crown batoidea. We suggest that the complex bundled enameloid present in other batoids is a convergent character that has appeared repeatedly during the evolution of batoids, probably as a mechanical adaptation towards moderate durophagous diets.

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INTRODUCTION

Chondrichthyes teeth are covered by a thin layer of hypermineralized tissue known as enameloid. In contrast to the ‘true’ enamel of tetrapod and sarcopterigian teeth, produced solely by ameloblasts of ectodermal origin, the enameloid is produced by a combination of ameloblasts and odontoblasts of ectomesenchymal origin (Gillis & Donoghue, 2007). The enameloid is mostly composed by hydroxy (fluor-)apatite crystallites and an organic matrix. The latter contains mainly collagen, synthesized by odontoblasts, and amelogenin-like proteins, produced by ameloblasts (Sasagawa, 2002; Sasagawa *et al.*, 2009; Sire, Donoghue & Vickaryous, 2009, and references therein).

The structure of the mineral phase of the enameloid in Chondrichthyes has an intricate hierarchical organization. The smallest repeating structural units are individualized elongated fluoroapatite crystallites [$Ca_5(PO4)_3F$], each formed by a number of hexagonal fluoroapatite unit cells. In a higher level of structural complexity, crystallites are arranged in parallel, forming tightly packed bundles, each with an envelope of organic matrix. Bundles with different orientations make assemblies that can be found in different layers, forming the whole enameloid layer of the teeth (Enax *et al.*, 2014).

The acquisition of these hierarchical structural levels has been progressive along the phylogeny of Chondrichthyans and traditionally has been related to the emergence of new feeding strategies in the group (Thies & Reif, 1985; Gillis & Donoghue, 2007; but see remarks in Enault *et al.*, 2015). Thus, cuspidate teeth of the grasping and swallowing dentitions

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of early chondricthyans that dominate Palaeozoic marine communities (such as *Leonodus* Mader, 1986; Xenacanthiforms, Ctenacanthiforms, Phoebodontiforms, and Symmoriiformes), possess an enameloid capping layer consisting of randomly oriented individual crystallites (single-crystallite enameloid, SCE), lacking any degree of higher microstructural differentiation (Gillis & Donoghue, 2007; Botella, Donoghue & Martínez-Pérez, 2009a). Exceptions to this 'general' pattern are found in certain Xenacanthiforms, which lack an enameloid cap (Hampe & Long, 1999; Gillis & Donoghue, 2007), and in the ctenacanth *Neosaivodus flagstaffensis* Hodnett, Elliott, Olson, Wittke, 2012, which shows crystallites associated into bundles parallel with the crown surface (PBE, see below; Guinot *et al.*, 2013). In many Hybodonts, a homogeneous layer of SCE is present (Reif, 1973; Gillis & Donoghue, 2007; Pla, Márquez-Aliaga & Botella, 2013; Enault *et al.*, 2015); however, some Mesozoic taxa with crushing dentitions developed a distinct two-layered enameloid consisting of an outer compact single-crystallite layer and an inner layer of SCE, with some crystallites organized into short, loosely defined bundles, usually perpendicular to the enameloid dentine junction (Cuny, Rieppel & Sander, 2001; Pla *et al.*, 2013; Enault *et al.*, 2015). In contrast, modern sharks (Selachimorpha) – i.e. non-batoid neoselachians – show a triple-layered enameloid consisting of: an inner layer of tangled bundled enameloid (TBE), with bundles of crystallites randomly oriented and adjacent to the dentine layer; an intermediate layer of parallel-bundled enameloid (PBE), with bundles of crystallites oriented parallel with the surface; and an outermost shiny layer enameloid (SLE), with single crystallites not arranged into bundles (Reif, 1973, 1977; for terminology, see also Cuny *et al.*, 2001). This complex microstructural differentiation has been considered as a pre-adaptation for the evolution of novel trophic strategies in this group (e.g. cutting, clutching, and grinding), increasing resistance to compressive forces and tensile strength that arise from these new feeding habits (Gillis & Donoghue, 2007; see also Preuschoft, Reif & Müller, 1974; Reif, 1978, 1979; Thies & Reif, 1985; Cappetta, 1986). According to Andreev & Cuny (2012), the acquisition of the triple-layered enameloid organization during the evolution of Selachimorphii occurred in consecutive steps from an ancestral single crystallite: beginning with the appearance of PBE (through several phases), followed by that of TBE, and considering the SLE as a remnant of the primitive SCE. In addition, the triple-layered enameloid of some derived shark teeth can either become more complex, adding some additional structures (reviewed in Enault *et al.*, 2015), or become simpler, by lacking specific components (Reif, 1973).

Noticeably, the organization of the enameloid in batoids, which represent more than half of all the extant neoselachians, has been poorly studied. Early studies on batoid enameloid have shown: (1) the lack of the triple-layered enameloid characteristic of Selachimorpha; and (2) the presence of great microstructural diversity in the group (Reif, 1977; Thies, 1982, 1983). Thus, Reif (1977) reported the presence of a thick cap of tangled fibred enameloid (TFE = TBE, after Cuny *et al.*, 2001) in *Rhina ancylostoma* Bloch & Schneider, 1801, whereas Thies (1983) found a SCE in some Early Jurassic *Spathobatis*; however, these findings remained partially neglected. As a result, it has been assumed that batoid enameloid was exclusively composed of a single layer of TBE (Gillis & Donoghue, 2007) or of SCE (Cuny *et al.*, 2009). A further debate emerged about whether this 'simplified' enameloid is a derived or a retained ancestral character for the group. These opposed interpretations have been influenced by both the placement of the batoidea in the chondrichthyan phylogeny, which has changed as the knowledge of this group has increased, and the different interpretations of the enameloid structure of some primitive batoids. Thus Underwood (2006), supported by the consideration that two putative Early Jurassic batoids (*Jurobatos* and *Doliobatis*) present a multilayered enameloid (Thies, 1983; Delsate, 2003; respectively), suggests that the single-layered enameloid of batoids is a derived character, reverted from the neoselachian triple-layered enameloid. This suggestion would agree with a view of batoids as a derived group of saw sharks and angel sharks (Hypnosqualea hypothesis, Compagno, 1977; de Carvalho, 1996; de Carvalho & Maisey, 1996; Shirai, 1992b, 1996); however, the placement of batoidea not as a derived group but as the sister group of all living sharks has been posteriorly well supported by both morphological and molecular data (Douday *et al.*, 2003; McEachran & Aschliman, 2004; Aschliman *et al.*, 2012). Additionally, the report of a multilayered enameloid in *Doliobatis* has been questioned; for example, Cuny *et al.* (2009) argued that the tissue interpreted as a TBE by Delsate (2003) is the dentine, and that *Jurobatos cappettai* Thies, 1993 is most likely not a batoid but a Galeomorpha (Thies, 1993). Therefore, Cuny *et al.* (2009) considered that only a SCE is present among basal batoids, and proposed that it is a retained plesiomorphic condition, as the SCE is also present in the successive sister groups of Neoselachii. The amalgamation of individual crystals into bundles would mark the appearance of Selachimorphii (Andreev & Cuny, 2012).

In contrast, several recent studies focusing on batoid enameloid oppose this perspective, claiming that a large number of batoid taxa present a layered bundled enameloid . Thus, in a study focused on the enameloid of stingrays, Enault, Cappetta & Adnet, 2013 (see also Cappetta, 2012) identified a double-layered enameloid in most Myliobatiformes, whereas a reduced single-layered enameloid was present only in some gymnurids and in the derived filter feeder mobulids. These facts, along with the identification of a double-layered enameloid in two non-myliobatoids taxa (*Raja* and *Rhynchobatus*), prompted Enault *et al.* (2013) to suggest that this is the plesiomorphic condition for batoids. Nevertheless, Enault *et al.* (2015) have later shown that, in fact, there are not two distinct layers in the enameloid of Myliobatiformes. These authors, however, still consider a complex enameloid as the ancestral batoid condition, based on the presence of a bundled multi-layered enameloid in several Mesozoic batoids (see Discussion).

In this work, we present a systematic analysis of the enameloid microstructure in current batoid fishes, broadening the span of the phylogeny of the group and focusing on non-myliobatiform taxa. The new data obtained in our analysis contrast with the recent interpretations of the plesiomorphic condition for batoidea (Enault *et al.*, 2013, 2015), which will be discussed here in a phylogenetic context.

MATERIAL AND METHODS

Taxa examined

Taxa examined in this study were selected to have a wide representation of the phylogeny of batoids (following that from Aschliman *et al.*, 2012). Thus, nine species belonging to the five major groups of batoids were chosen to analyse their enameloid microstructure. Skates (Rajoidei) are represented by *Raja clavata* Linnaeus, 1758; *Atlantoraja platana* Günther, 1880; *Sympterygia acuta* Garman, 1877 and *Rioraja agassizii* Müller & Henle, 1841. *Torpedo marmonata* Risso, 1810 is chosen to represent the electric rays (Torpedinoidei), *Plathyrinoidis triseriata* Jordan & Gilbert, 1880 exemplifies the thornbacks (Platyrhinoidei), *Dasyatis americana* Hildebrand & Schroeder, 1928 represents the stingrays (Myliobatoidei), and polyphyletic 'guitarfishes' are represented by *Rhinobatos producuts* Ayres, 1854 (Rhinobatoidei) and *Rhyna ancylosotma* (Rhinoidei). In the case of species with sexual dimorphism we have studied both male and female teeth when they were available, as indicated for each taxa.

All the teeth were loaned by the museum El Cau del Tauró (L'Arboc, Tarragona, Spain). Teeth were removed from prepared jaws, selecting those placed at intermediate positions of the dental family. All the teeth studied are deposited in the Museum of Geology at the University of Valencia: MGUV 27749–60.

Analysis of enameloid structure

For the study of enameloid microstructure, teeth were embedded in Canada balsam at 120°C for 2 h prior to being polished with a mix of carborundum (800 and 1200 μm) and water, following the chosen plane of section (longitudinal or transversal). Afterwards, the sections were etched. Two protocols were tested for the etching of the teeth. The first is the most commonly used for the study of fossil sharks (e.g. Cuny & Risnes, 2005; Gillis & Donoghue, 2007; Botella *et al.*, 2009a), where teeth were etched in HCl 10% for 5–10 s. The second treatment seems more accurate for removing the organic matrix present in recent material (Enault *et al.*, 2013), and teeth were etched in hydrogen peroxide for a time period ranging from a few hours up to 3 days. Both treatments were used separately in a number of specimens used as controls (including triple-layered selachimorphy teeth). In general, satisfactory results can be obtained for both methods because they do not show significant differences; nevertheless, the clearest images were visualized with hydrogen peroxide, so this treatment was mostly used in our study. Each sample was re-polished and etched as many times as necessary to expose the enameloid microstructure. Additionally, some teeth were broken for the direct observation of a fresh fracture. The broken surfaces were etched as described above. The analysis and photography of ground sections were performed on a Hitachi S–4100 scanning electron microscope of the Microscope Service of the University of València. For SEM analysis, teeth were coated with gold and palladium alloy.

RESULTS

Skates

Atlantoraja platana Günther, 1880 (Fig. 1A–D)

The dentition of this species displays sexual heterodoncy. In females, the teeth are of the crushing type, with flat crowns and little pronounced rounded cusps, whereas in mature males dignathic heterodoncy is present in addition with monocuspidate teeth (with long and sharp cusps) in the central region and teeth with flattened crowns in the distal regions (Rangel *et al.*, 2014). In both sexes, teeth of *Atlantoraja* are covered by a thin homogeneous monolayer of SCE that visibly differs from the underlying dentine core. No microstructural differentiation is observed across the enameloid layer (Fig. 1B, C). At higher magnification, individual crystallites are clearly discernable. They are elongate, ~1 μm in length, and appear to be randomly oriented (Fig. 1B, D).

Raja clavata Linnaeus, 1758 (Fig. 1E–K)

As in *A. platana*, both sexual and dignathic heterodoncy is present in this species. In the pointed clutching-type teeth of male upper jaws, the enameloid layer is wider in the apex of the cusp

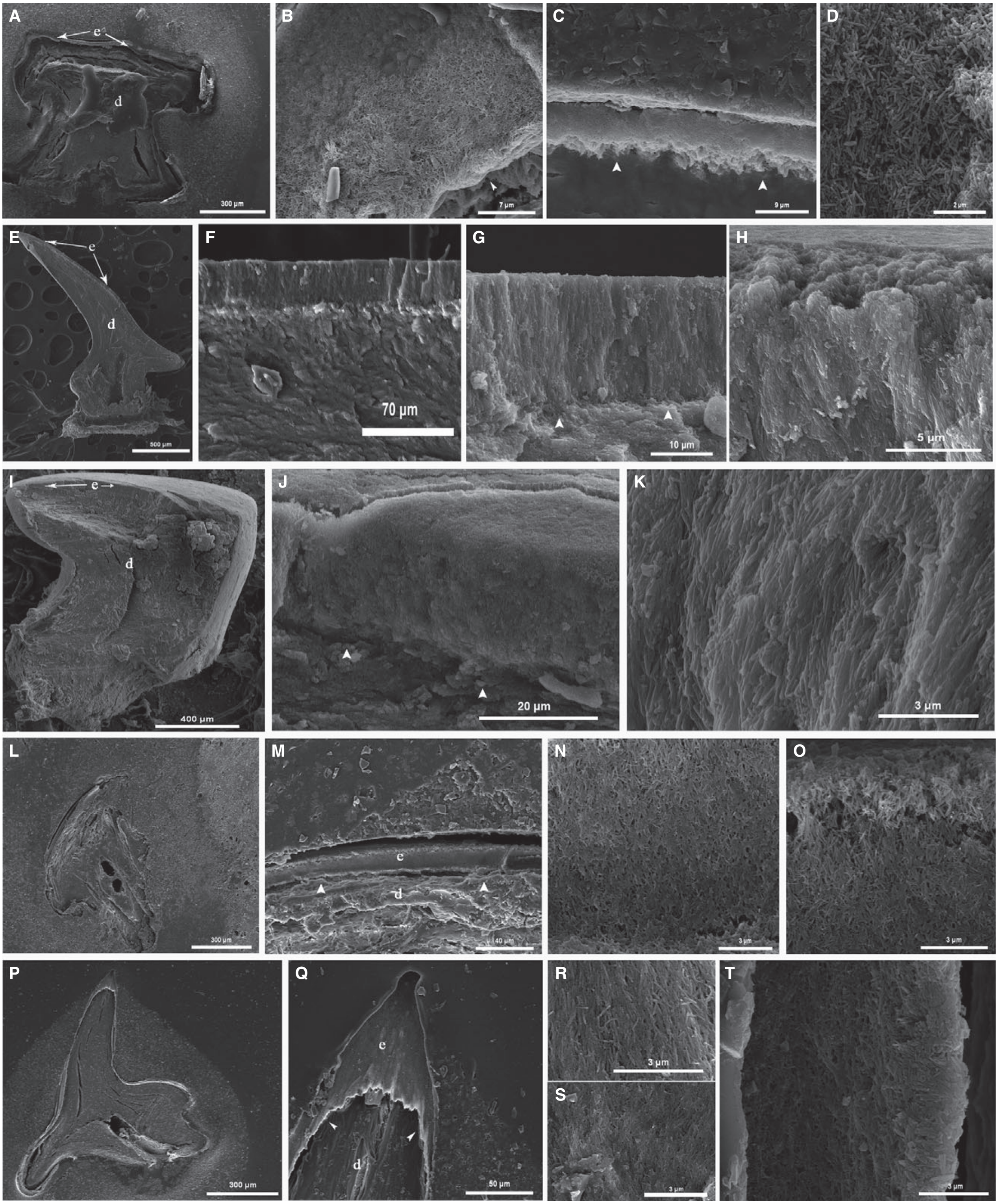


Figure 1. Scanning electron micrograph of batoid enameloid: Rajoidei. A–D, *Atlantoraja platana*, tooth of a female, MGUV 27749, etched for 3 h in H_2O_2. A, overview of tooth in longitudinal section; B, enameloid layer at the tooth edge; C, aspect of the enameloid layer in the centre of the crown – a well-defined but irregular enameloid–dentine junction (EDJ) is present; D, detail of the randomly organized individual crystallites. E–H, *Raja clavata*, tooth of a male, etched for 1 day in H_2O_2, MGUV 27750. E, general view of the tooth, showing the enameloid capping layer, the dentine core, and a central pulp cavity in a fresh fracture; F, detail of the enameloid layer and the underlying dentine tissue; G, H enameloid crystallites are highly compacted and arranged perpendicular to the EDJ. I–K, tooth of a female *Raja clavata*, etched for 1 day in H_2O_2, MGUV 27751. I, overview of the fresh fracture of the tooth showing the enameloid layer and the dentine core; J, general aspect of the enameloid layer; K, detail of the enameloid crystallites, highly compacted and arranged perpendicular to the crown surface. L–O, tooth of a male *Rioraja agassizii*, etched for 3 h in H_2O_2, MGUV 27753. L, complete tooth, showing the enameloid layer and the dentine core; M, detail of the whole layer of single-crystallite enameloid; N, detail of the individual fluorapatite crystallites randomly oriented in the inner part of the layer; O, N, detail of the individual fluorapatite crystallites randomly oriented in the outermost part of the layer. P–T, tooth of a male *Sympterigia acuta*, etched for 3 days in H_2O_2, MGUV 27754. P, overview of an embedded tooth; Q, enameloid cap at the cusp of the tooth, showing the presence of a sharp enameloid–dentine junction EDJ; R, detail of the enameloid crystallites, highly compacted and predominantly oriented perpendicularly to the enameloid–dentine junction; S, detail of the crystallites near the apex surface; T, detail of the enameloid layer on the lateral part of the cusp with the enameloid crystallites randomly oriented in the cusps. Arrowheads point to the EDJ; d, dentine; e, enameloid. Scale bars: A, L, P, 300 μm; B, 7 μm; C, 9 μm; D, 2 μm; E, 500 μm; F, 70 μm; G, 10 μm; H, 5 μm; I, 400 μm; J, 20 μm; K, N, O, R, S, T, 3 μm; M, 40 μm; Q, 50 μm.

and becomes thinner towards the base (Fig. 1E). In the rounded, crushing-type teeth of female lower jaws, the enameloid layer is homogeneous all over the superior part of the crown surface, but is considerably thinner in the laterals parts (Fig. 1I). In all teeth studied the enameloid consists of SCE. Single crystallites are compacted and arranged perpendicular to the enameloid–dentine junction (EDJ) in almost the complete layer (Fig. 1F, G, H, J, K), although in the outermost surface the orientation of crystallites changes, appearing parallel with the tooth surface (Fig. 1H, J). The enameloid crystallites are elongate and measuring ~2 μm in length.

Rioraja agassizii Müller & Henle, 1841 (Fig. 1L–O)
Sexual dimorphism present. Only female teeth were accessible: they are of the grasping type, with rounded crowns (Fig. 1L). The enameloid is very similar to that of *Atlantoraja*. The SCE is homogeneously composed of randomly oriented enameloid crystallites of ~1 μm in length, and they are elongate with rounded ends (Fig. 1N, O). A sharp EDJ is apparent, and the enameloid crystallites seem to be less compacted near this junction (Fig. 1M).

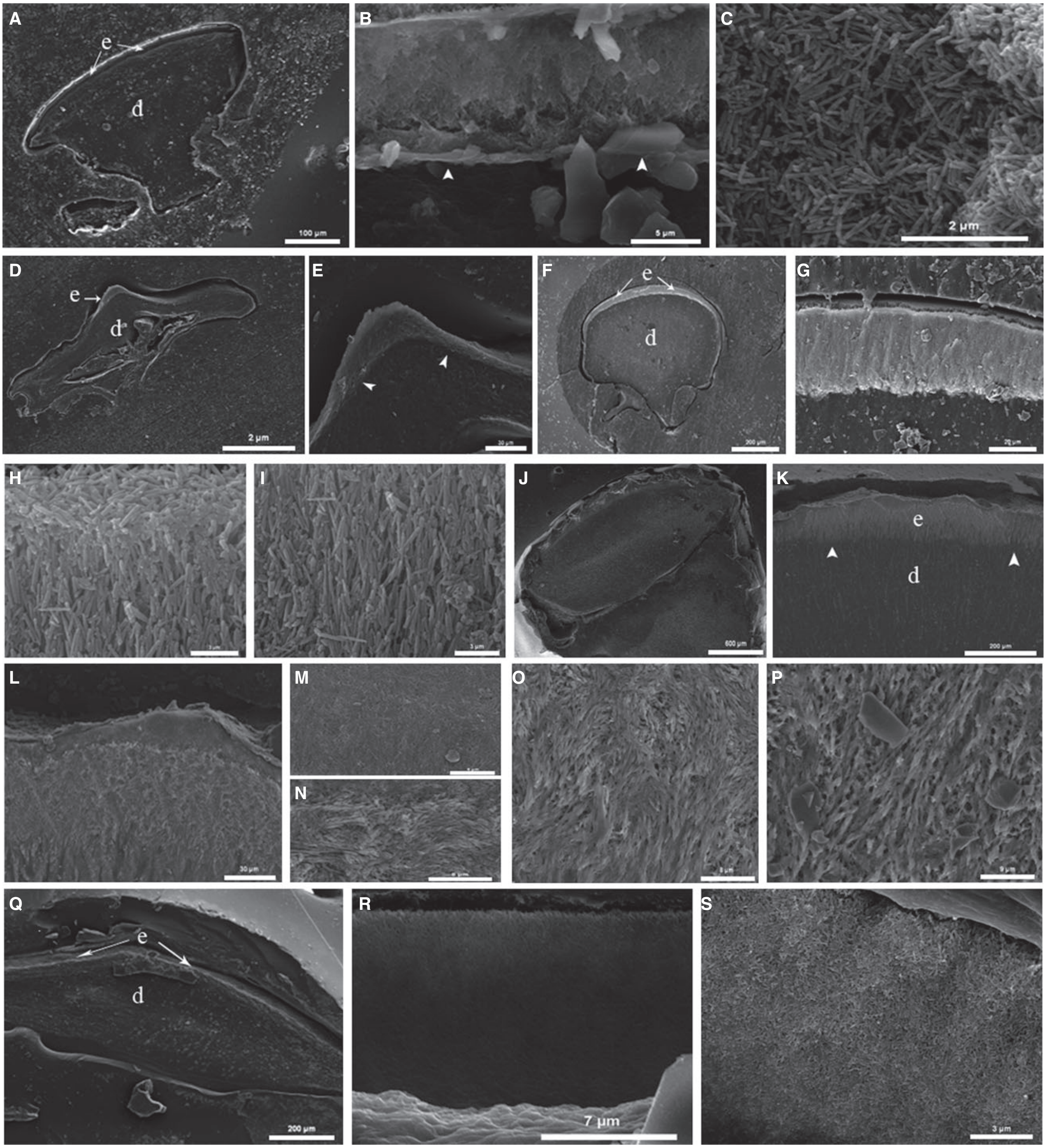
Sympterygia acuta Garman, 1877 (Fig. 1P–T)
Sexual dimorphism is present. Only teeth of males were accessible for our study. They are cuspidate with a single pointed cusp (Fig. 1P). A SCE layer caps the crown of the teeth. The width of the layer changes laterally, being widest at the cusp apex and becoming thinner towards the base (Fig. 1Q, T). The orientation of enameloid crystallites also varies depending on the area, thus near the top of the cusp hydroxiapatite crystallites are highly compacted and are predominantly oriented perpendicular to the teeth surface (Fig. 1Q–S), whereas in the lateral side of the cusp and in the base of the crown crystallites are less compacted and randomly oriented (Fig. 1T). Noticeably, the size and shape of the crystallites also vary depending on their position: those in the tip of the crown are elongated and 2 μm in length (Fig. 1R, S), whereas crystallites near the base are more round in shape and measure less than 1 μm in length (Fig. 1T).

THORNBACKS

Platyrhinoidis triseriata Jordan & Gilbert, 1880 (Fig. 2A–C)
Sexual heterodonty is apparent, with a relatively high, elongated principal cusp on the teeth of males, versus lower cusps in females. Only female teeth were analysed here. Their crushing-type teeth show a layer of SCE lacking any type of microstructural differentiation. The thickness of the layer is homogeneous through the flattened crown of the teeth (Fig. 2A). Individual crystallites appear to be randomly distributed and compacted, although towards the EDJ the crystallites are less compacted (Fig. 2B). The hydroxipatite crystallites are elongate and measure 1 μm in length (Fig. 2C).

ELECTRIC RAYS

Torpedo marmonata Risso, 1810 (Fig. 2D, E)
No dental sexual dimorphism has been described in this species. The small clutching teeth of this species present a single-pointed central cusp. To



observe the enameloid layer, the minute size of the available teeth made the etching process difficult. Nevertheless, although individual hydroxiapatite crystallites are not discernible, a thin enameloid layer is histologically distinguishable from the underlying dentine core. It clearly lacks the tubule-rich texture of dentine matrix. The enameloid layer is homogenous and reveals no indication of any degree of microstructural differentiation within it, and thus the presence of a monolayer of SCE, similar to that present in Rajiformes, can be inferred.

GUITARFISHES

Rhinobatos productus Ayres, 1854 (Fig. 2F, I)
Sexual heterodonty is shown by low cusps on the anterior and lateral side of male teeth, versus a

Figure 2. Scanning electron micrograph of the enameloid in batoids: Platyrhinoidei (A–C); Torpedinoidei (D–E); Rhinobatoidei (F–I); Rhinoidei (J–P); and Dasyatidae (Q–S). A–C, tooth of *Platyrhinoidis triseriata* embedded and etched for 1 day in H_2O_2, MGUV 27755; A, overview of the whole tooth; B, enameloid layer and the enameloid–dentine junction (EDJ); C, detail of the randomly oriented hydroxiapatite crystallites. D–E, embedded tooth of *Torpedo marmonata*, etched for 2 s in 10% HCl, MGUV 27756; D, general view of the polished surface of the tooth; E; detail of (D), individual crystallites are not discernable in MGUV 27756 but a monolayer enameloid is clearly distinguishable from the dentine below. F–I, tooth of the guitarfish *Rhinobatos productus*, etched for 5 s in 10% HCl, MGUV 27757; F, embedded tooth showing the enameloid layer and the dentine core; G, detail of the whole enameloid layer; H, crystallites randomly distributed in a plane parallel with the surface of the tooth, whereas in the inner part the crystallites are more compacted and are distributed in a preferred orientation, perpendicular to the dentine; I, crystallites near the EDJ; J–P, teeth of *Rhyna ancylostoma*, etched for 5 s in 10% HCl, MGUV 27758; J, embedded tooth in longitudinal section; K, general view of the EDJ; L, aspect of the enameloid in a crest of the crown, organized in two different layers, with the limit between the layers clearly defined; M, detail of (L) showing the outer layer composed of randomly oriented crystallites; N, detail of (L) showing the limit between the outer single-crystallite enameloid and the inner bundled-crystallite enameloid layers; O, inner layer showing bundles in an interwoven texture; P, bundles near the EDJ. Q–S, teeth of a female individual of *Dasyatys americana* etched for 3 h in H_2O_2, MGUV 27759; Q, embedded tooth in longitudinal section; R, detail of the whole enameloid layer showing no microstructural differentiation; S, enameloid crystallites randomly oriented. Arrowheads point to the EDJ; d, dentine; e, enameloid. Scale bars: A, 100 μm; B, 5 μm; C, D, M, N, 2 μm; E, G, L, 30 μm; F, K, Q, 200 μm; H, I, S, 3 μm; J, 600 μm; o, 8 μm; P, 9 μm; r, 7 μm.

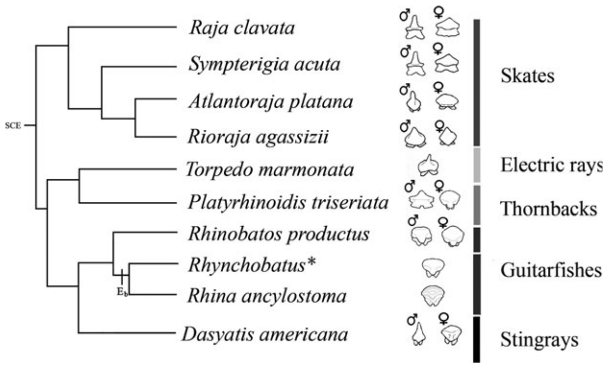


Figure 3. Phylogeny of the extant batoid fishes considered in this study, with the distribution of tooth enameloid microstructure. **Rhynchobatus** has not been sampled here, and the presence of a bundled enameloid in this species is based on Cappetta (2012). Only *Dasyatys americana* has been sampled here as a representative of the stingrays, but the work of Enault *et al.* (2015) indicated that a classical single-crystallite enameloid (SCE) is present in other Myliobatiformes. Phylogenetic interrelationships are based on Aschliman *et al.* (2012). Skates are recovered as sister group to all other extant batoids and 'guitarfishes' are recovered as a polyphyletic group, with sawfishes (not represented here) nested within the clade considered here. E_b mark the occurrence of bundled-crystallite enameloid. Illustrations of teeth are not drawn to scale.

slightly arched transverse keel in females (Herman *et al.*, 1997). Only female jaws were available for the study. Their crushing-type teeth possess a thin layer (i.e. 34 μm thick in a tooth of 572 μm in depth) of SCE capping their crown surface (Fig. 2F, G). In the outermost part of the enameloid layer, crystallites are randomly distributed in a plane parallel with the surface of the tooth, whereas in the inner part crystallites are more compacted and are in a preferred orientation perpendicular to the dentine core (Fig. 2G, H). Towards EDJ, the crystallites are more randomly oriented and less compacted than in the rest of the layer (Fig. 2I) – a pattern that is also found in the tooth edges. The enameloid crystallites are elongate and reach up to 3 μm in length; they are easily differentiated individually.

Rhina ancylostoma Bloch & Schneider, 1801 (Fig. 2J–P)

Sexual dimorphism has not been reported in the teeth of this species. The crushing teeth of *Rhina* are covered with a thick layer of enameloid that becomes progressively thinner towards the edges (Fig. 2J). The enameloid is organized into two different layers (Fig. 2L–P). The limit between the layers is very well defined (Fig. 2L, N). The outer layer is composed of densely packed crystallites that are randomly oriented (Fig. 2K–M). The inner layer exhibits complex organization. In the outer part of this layer, adjacent to the SCE layer, crystallites appear to be arranged in short bundles of interwoven texture (Fig. 2L, N, O). In the innermost part, reaching the EDJ, bundles are longer and generally oriented perpendicular to the EDJ, although they are crossed by bundles oriented in other planes (Fig. 2L, P). The border with the underlying dentine is uneven, with dentine tubules penetrating into the enameloid layer. Crystallites within a bundle show similar orientation. The relative thickness of the enameloid layers varies along the teeth: the outer SCE layer is thicker in the ridges that ornament the crown, but becomes thinner in the spaces between ridges, whereas the inner layer has a more continuous thickness (~50–60 μm in the teeth studied). Therefore, in some parts of the crown (e.g. the spaces between ridges and the labial and lingual shoulder of the crown; Fig. 2N) the outer layer is reduced or absent and most of the enameloid layer is formed by short bundles of woven texture (Fig. 2O). The hydroxiapatite crystallites are differentiated individually and are elongate, with a total length of 1 μm.

STINGRAYS

Dasyatis americana Hildebrand & Schroeder, 1928 (Fig. 2Q–S)
Sexual dimorphism is present in this species, but only the teeth of female were available. A thick layer of SCE caps the surface of their crushing teeth (Fig. 2Q). The hydroxiapatite crystallites are randomly organized along the entire layer and are highly compacted (Fig. 2R); however, each crystallite can be easily distinguished (Fig. 2S). The enameloid crystallites are elongate with pointed ends, and are of no more than 1 μm in length.

DISCUSSION

BATOID ENAMELOID MICROSTRUCTURE

Our SEM analysis of the enameloid microstructure in the teeth of a selection of taxa covering the phylogeny of batoid fishes provides evidence of the presence of a superficial layer of SCE capping the teeth of most of the species studied, i.e. in the Rajoidei (*Raja clavata*, *Atlantoraja platama*, *Sympterygia acuta*, and *Rioraja agassizii*), Platyrhinoidei (*Platyrhina triseriata*), Rhinobatoidei (*Rhinobatos productus*), Torpedinoidei (*Torpedo marmonata*), and Myliobatoidei (*Dasyatis americana*). In all of these species, the enameloid consists of a homogenous monolayer made of individualized crystallites with no apparent superior microstructural differentiation (i.e. bundles or fibres; Figs 1 and 2A–I, Q–S). The only exception to this general pattern was found in the enameloid of the Rhinoidei *Rhina ancylostoma*, where crystallites of the outermost part of the layer are individualized and highly compacted (SCE), whereas in the innermost part single crystallites are organized into bundles with variable orientation (Fig. 2J–P). As noted above, in some parts of the teeth the SCE component is reduced or is not present, and the enameloid layer is mostly formed by short entangled bundles of woven texture (Fig. 2O). It is probably this fact that induced Reif (1977) to consider that only a TFE (TBE) is present in *Rhina ancylostoma*. The organization of the enameloid of *Rhina ancylostoma* is in fact very similar to that found recently in the Late Cretaceous batoids *Parapalaeobates* cf. *atlanticus* Arambourg, 1952 and *Ptychotrygon* sp. (Enault *et al.*, 2015).

Recent studies (Cappetta, 2012; Enault *et al.*, 2013) have noted that the crystallites of enameloid in batoid fishes are poorly individualized and that, moreover, the morphology of the bundles of ‘fibres’ of batoids differs from the bundles of sharks, which are composed of microcrystallites. Our analysis, however, evidenced that well-individualized crystallites are clearly discernable in all batoid taxa studied. Even in the case of the double-layered enameloid found in *Rhina ancylostoma*, the bundles of the inner layer are composed of single crystallites that remain individually discernable (Fig. 2O, P).

The cases of *Raja* and *Dasyatis* deserve special consideration, as the structure of their enameloid found in our analysis differs from other previous interpretations. Thus, according to Enault *et al.* (2013), the enameloid of *Raja clavata* and most myliobatiforms, including *Dasyatis*, consists of at least two layers: an outer, very compact layer of poorly individualized crystallites, without clear microstructure; and an inner layer formed by parallel ‘bundles of fibres’ that are perpendicular to the crown (complex B, *sensu* Enault *et al.*, 2013). Their figures do not allow for the clear identification of any kind of bundles (or fibres), however, and the existence of the two layers is doubtful (see Enault *et al.*, 2013: figs 2A, C–K). In fact, Enault *et al.* (2015) have later stated that only a SCE layer is present in Myliobatiformes, noting that their previous description of two distinct layers in the enameloid of several Myliobatiforms was a misinterpretation resulting from the poor resolution of some scanning electron microscopy (SEM) micrographs. In our opinion, the identification of a double-layered enameloid in other taxa studied by Enault *et al.* (2013) is also dubious, and needs further discussion. Thus, the differences observed in the innermost part of the enameloid layer in the specimen of *Raja clavata* described in Enault *et al.* (2013: fig. 2A) probably result from the presence of abundant dentine tubules extending up to the enameloid, across the EDJ (an usual character of chondrychthyan enameloid; see Gillis & Donoghue, 2007), or are signs of the reticular structure of the EDJ. In order to better support our results, we analysed several teeth of both females and males and both upper and lower jaws of *Raja clavata*. In all of the specimens studied the enameloid consists of a SCE monolayer lacking any higher microstructural differentiation. Single crystallites are clearly individualized and arranged perpendicular to the EDJ, except in the very outermost part of the layer where they become oriented parallel with the tooth surface. Interestingly, specializations in the SCE, similar to that found here in *Raja*, have been found in some non-neoselachian sharks with uncertain phylogenetic affinities. For example, teeth of the cutting-clutching dentitions of *Pseudodalatias* show a SCE very similar to that found in *Raja*, with highly compacted crystallites oriented perpendicular to the surface in the inner part of the layer, and arranged parallel with the surface in the very outermost part of the layer (Reif, 1973; Botella *et al.*, 2009b). *Pseudodalatias* is an enigmatic tooth-based genus of Triassic chondrichtyes that has been considered as a presumptive stem batoid, or perhaps a stem neoselachian (Botella *et al.*, 2009b; Andreev & Cuny, 2012).

A double-layered enameloid was also identified by Enault *et al.* (2013) in extant *Rhynchobatus* sp. The picture depicting this case (Enault *et al.*, 2013: fig. 2B) shows an enameloid layer with two different zones: a compact outer thick zone and an inner zone with thick enameloid ‘pillars’ intermingled with dentine, but the magnification of the figure prevents the unambiguous identification of crystallite bundles in the inner layer. However, Cappetta (2012) provide more detailed SEM images of *Rhynchobatus* [*R. pristinus* (Probst, 1877), middle Miocene, France] where the presence of a bundled enameloid, with parallel bundles normal to the EDJ in the inner part, and with interwoven bundles towards the crown surface, is more clear. Thus, although more precise illustrations of the enameloid of this genus are required to support their observations conclusively, the double-layered enameloid identified in *Rhynchobatus* by Cappetta (2012) and Enault *et al.* (2013) clearly differ from the SCE present in the other ‘rhinobatoid’ taxa studied here, *Rhinobatos productus*, and seems somewhat similar to the ‘double-layered’ enameloid found in *Rhina ancylostoma*. Remarkably, the genera *Rhina* and *Rhynchobatus* have long been included in a single family, Rhinidae (=Rhynchobatidae) by several authors (e.g. Müller & Henle, 1841; or more recently Compagno & Last, 1999). Moreover, most recent molecular phylogenies (Aschliman *et al.*, 2012; Naylor *et al.*, 2012) placed them as sister groups nested deeply within ‘guitarfishes’, but closely related to sawfishes. Accordingly, *Rhina* (Rhinidae) and *Rhynchobatus* (Rhynchobatidae) are included in a monophyletic order, Rhinopristiformes, together with Rhinobatidae and Pristidae (Naylor *et al.*, 2012).

PHYLOGENY OF BATOID TOOTH ENAMELOID

When the enameloid microstructure data for the taxa studied here were mapped on a recent molecular phylogeny of extant batoid fishes (Fig. 3), it provided phylogenetic support to consider the monolayer SCE lacking microstructural differentiation as the primitive condition for batoids. The bundled enameloid in *Rhina ancylostoma* – and presumably in *Rhynchobatus* (see remarks above) – must be considered as a derived character that appeared during the diversification of Rhinopristiformes (*sensu* Naylor *et al.*, 2012). Thus, as recent phylogenetic analysis placed batoids as the sister group of all other neoselachians (Douday *et al.*, 2003; Maisey, Naylor & Ward, 2004; McEachran & Aschliman, 2004; Winchell, Martin & Mallatt, 2004; Aschliman *et al.*, 2012; Naylor *et al.*, 2012), and a SCE is the primitive state for all non-neoselachian groups (Reif, 1973; Gillis & Donoghue, 2007; Botella *et al.*, 2009a), the idea that Batoidea has retained the plesiomorphic condition of enameloid microstructure seems the most parsimonious option, as was suggested by Cuny *et al.* (2009) based on the presence of a SCE in some basal batoids.

Our phylogenetic interpretation contrasts with recent hypotheses that consider double-layered bundled enameloid as the plesiomorphic condition for batoids (Enault *et al.*, 2013, 2015); however, the proposal of Enault *et al.* (2013) was established on a misinterpretation of the enameloid microstructure of several taxa. The enameloid that was interpreted as a bundled two-layered enameloid in some myliobatiforms and in *Raja clavata* is in fact a SCE (see Enault *et al.*, 2015 and below). On the other hand, the suggestion of Enault *et al.* (2015) is based on the finding of a complex bundled enameloid in several fossil batoids. These authors clearly showed the presence of bundled enameloid in two different units of the teeth of *Pytchotrygon* sp. (Turonian, Upper Cretaceous, Morocco), in ‘rhinobatoids’ *Belemnobatis* sp. (Aptian Lower Cretaceus, France), and in *Parapalaeobates* cf. *atlanticus* (Campanian, Upper Cretaceous, Morocco); in contrast, a SCE was found in other Rhinobatoid (*Hypsobatis weileri* Cappetta, 1992, Maastrichtian, Late Cretaceous, Morocco) and in some Myliobatiformes. Based on the oldest specimen that they investigated (*Belemnobatis* sp.), they suggest that the ancestral batoid enameloid exhibited some degree of complexity; however, Enault *et al.* (2015) overlooked the fact that a SCE had been recognized previously in older taxa, i.e. the Early Jurassic *Spathobatis moorbergensis* Thies, 1983 and *Spathobatis* sp. (Thies, 1983), the Middle Jurasic *Belemnobatis aominensis* Cuny, Srisuk, Khamha, Suteethorn & Tong, 2009, and probably, according to Cuny *et al.* (2009), in the oldest known batoid, the Early Jurassic *Doliobatis weisi* Delsate & Candoni, 2001. In any case, identifying plesiomorphic conditions based on the stratigraphic position of taxa is problematic, and the phylogenetic relationships of guitarfish-like batoids from the Jurassic and the Lower Cretaceous are still unclear (Underwood, Mitchell & Veltkamp, 1999; Underwood, 2006; Cuny, 2009). Only a few phylogenetic studies of batoids have incorporated these extinct taxa (essentially *Spathobatis* and *Belemnobatis*, known from articulated specimens). Furthermore, although it is well established that they present a number of significant differences that separate them from extant rhinobatoids (Maisey, 1984; Aschliman, 2011; and refereneces therein), their position within batoid phylogenies vary among different authors. Thus, Maisey (1984) tentatively placed Torpediformes at the basal level of the batoid phylogeny and a trichotomy containing (*Spathobatis* + *Belemnobatis* + *Pristis*) as the sister group of the remaining batoids. Brito & Seret (1996) considered (*Rhynchobatus* + *Rhina*) as the most basal batoid representatives, and placed the group (*Spathobatis* + *Belemnobatis*) as sister to all other batoids. In Underwood *et al.* (1999), sclerorhynchoidei and (*Spathobatis* + *Belemnobatis*) are recovered as successively distant rootward sister groups to all other batoids, except torpediniforms. This aspect of the topology resembles that of Maisey (1984), except for the placement of *Pristis*. Finally, more recently, Claeson, Underwood & Ward (2013) considered *Sclerorhynchus* and *Spathobatis* as two consecutive stem-batoid groups, with *Spathobatis* as sister to crown batoids. Similarly, the systematic position of *Ptychotrygon* remains controversial, even at a subordinal (or upper) level (Cappetta, 2012; see also Underwood, 2006; Kriwet, Nunn & Klug, 2009). In consequence, the plesiomorphic condition of batoid enameloid remains currently unresolved, as (1) both a SCE and a complex layered bundled enameloid have been found in early batoids, and (2) the exact phylogenetic placement of these taxa within batoids is uncertain.

CONCLUSION

We have studied the enameloid microstructure in a selection of taxa, trying to cover the phylogeny of living batoids. Our SEM analysis demonstrated that a SCE lacking microstructural differentiation is the most widespread condition among extant batoids, and is probably plesiomorphic at least for crown batoidea, and is not a derived character of the Myliobatiformes, as suggested by Enault *et al.* (2015). In fact, a homogenous SCE monolayer with very conspicuous individualized crystallites is present, capping the teeth of all species studied, with the Rhinoidei *Rhina ancylostoma* being the only exception. Notably, our studies evidenced that SCE enameloid is present in the teeth of both females and males of the species *Raja clavata*, in contrast with previous interpretations asserting the existence of bundled double-layered enameloid in this taxa.

The double-layered enameloid of *Rhina ancylostoma* has an outermost part in which crystallites are individualized and highly compacted and an innermost part where crystallites are organized into bundles of variable orientation. This microstructure is similar to that described in extant *Rhynchobatus* sp. (Cappetta, 2012), which is placed as sister group of *Rhina* according to most recent molecular phylogenies (Aschliman *et al.*, 2012; Naylor *et al.*, 2012). Moreover, the organization of the enameloid of *Rhina ancylostoma* is also very similar to that found in several distantly related taxa, such as the fossil batoids *Parapalaeobates* cf. *atlanticus* and *Ptychotrygon* sp. (Enault *et al.*, 2015). This suggests that the complex bundled enameloid present in some batoids is a convergent character that appeared more than once during the evolution of batoids, probably as a mechanical adaptation towards moderate durophagous diets.

The plesiomorphic condition of enameloid for the whole group of batoids, including stem lineages, remains unclear, because the phylogenetic position of most guitarfish-like batoids from the Jurassic and the Lower Cretaceous remains unresolved (Underwood *et al.*, 1999; Underwood, 2006; Cuny, 2009), and both a SCE and a complex layered bundled enameloid are present in these early batoids. Therefore, further studies on both the diversity of the enameloid microstructure in early batoids and on their phylogenetic status are needed to clarify this question.

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