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APPLICATION OF SCANNING ELECTRON MICROSCOPY TO THE STUDY OF SHARK DERMAL DENTICLES

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Abstract

Clear, high resolution scanning electron micrographs of shark dermal denticles are essential to allow the study of their morphology and micro-relief. Various techniques were tried to remove mucus and adventitious debris, including KOH, trypsin enzyme, and ultrasonication. In most species examined the best results were obtained with enzyme treatment followed by ultrasonication. For scanning electron microscopic examination it was found that 15 nm gold coating, or more, and 10 kV, or less, had to be employed to reduce charging of the denticles.

KEY WORDS: Shark dermal denticles; specimen preparation; Scanning Electron Microscopy (SEM).

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Introduction

Since Müller and Henle (1838-1841) shark dermal denticles have been studied as a taxonomic character. Such studies were carried out under a dissecting microscope, and mainly described the dermal denticle morphology and micro-relief of different species. Reif (1973, 1974, 1978a, 1978b, 1979, 1980) first used scanning electron microscopy (SEM) for studying shark dermal denticles, describing their ontogeny, morphogenesis, structure, and wound healing pattern. Dingerkus and DeFino (1983) first used SEM morphology of shark dermal denticles to study the interrelationships between species and genera of sharks. None of these papers described in detail how denticle samples were prepared for the SEM.

The sharks' skin and dermal denticles are coated with a layer of mucus. This mucus dries upon the denticles and leaves a layer of debris when they are prepared for the SEM (fig. 1). Sand and other material may also be glued onto the denticles by this mucus. The object of our studies herein presented is to describe the removal of such debris and adventitious material from shark dermal denticles and the techniques used to obtain clean, clear high resolution photomicrographs of denticles with the SEM.

Materials and Methods

Skin samples were obtained from freshly caught sharks, taken off the Bimini Islands, Bahamas. Species included: lemon shark (Negaprion brevirostris); tiger shark (Galeocerdo cuvieri); great hammerhead shark (Sphyrna mokarran); bull shark (Carcharhinus leucas); Caribbean reef shark (Carcharhinus perezi); blacktip shark (Carcharhinus limbatus); blacknose shark (Carcharhinus acronotus); nurse shark (Ginglymostoma cirratum); sharpnose shark (Rhizoprionodon porosus); and American stingray (Dasyatis americana). Complete collection and specimen deposition data are available from the senior author. Skin samples were taken from the right dorso-lateral side of the animals, between the shoulder and first dorsal fin. A sample of about 5 cm square was removed, excess flesh was scraped off with a fileting knife, and then washed in sea water to

remove blood, etc. Specimens were pinned out on a styrofoam block, flesh-side up, to reduce shrinkage and buckling of the skin sample, and air dried in a place where a fan was blowing on them to speed drying. A piece of plastic bag was placed under the skin samples when they were pinned out, so that they would not adhere to the styrofoam block. This method of drying produces some curling of the sample's edges. In order to obtain a reasonably flat sample for the SEM, these curled edges had to be cut off. For most samples, a pair of scissors was employed, however in the case of thick skin samples from large sharks, a hacksaw had to be employed. Most of the samples were subdivided in the laboratory to study the effects of various cleaning techniques on the same piece of skin. Average size of the samples placed in the SEM was 1 cm square. Most of the samples were cut to this size with a pair of scissors, but again in the case of thick skin from large sharks a hacksaw had to be employed.

To remove mucus and other debris from the denticles, five different procedures were used: ultrasonication; enzymatic digestion, with and without ultrasonication; and KOH, with and without ultrasonication. Enzymatic digestion was performed using a solution of 30 cc saturated sodium borate (aqueous) and 70 cc distilled water, to which 1 g trypsin enzyme (4X pancreatin, lab grade) was added, for 1 h at room temperature; followed by two 15 min washings in distilled water and a final 30 min wash in 70% ethyl alcohol. Samples were then air-dried. During drying a light weight was placed on the samples (which were surrounded by pieces of plastic bags to prevent adherence) to hold them flat. Base maceration was done by placing skin samples in 1% aqueous KOH. Specimens were then washed and dryed as for the enzymed specimens.

The samples of untreated skin and of the five cleaning treatments were then prepared for SEM observation. Samples were mounted on 2.54 cm diameter aluminum pin-type specimen stubs, that had been ethyl alcohol cleaned, using pieces of "Fun-Tak" (or "Permabond"--National Starch & Chemical Corp., Englewood, NJ) as the adhesion agent between stub and specimen. The permabond was applied to the undersurface of the skin sample and silver painted lightly at the juncture of permabond and skin. The skin sample was then placed on the stub (permabond-side down) and pressed down firmly with large forceps only on opposite edges of the skin to give as even a surface as possible. Silver paint was then applied lightly around the edges ensuring electrical conductivity between the skin and the stub. The mounted samples were coated with 10 or 15 nm of gold in a Polaron E5100 sputter coater; thickness was measured with an attached Polaron film thickness monitor. Argon gas was used to flush the chamber. The coater was operated at 1.8 kV and approximately 18 ma (controlled by the argon gas flow). Coated specimens were viewed in an AMRay Model 1600T (equivalent) SEM at 10 or 20 kV, using a tungsten filament and a 100 µm final aperture. Micrographs were taken on Kodak Royal Pan 10.16 x 12.7 cm negative sheet film (RP 4141 estar thick base).

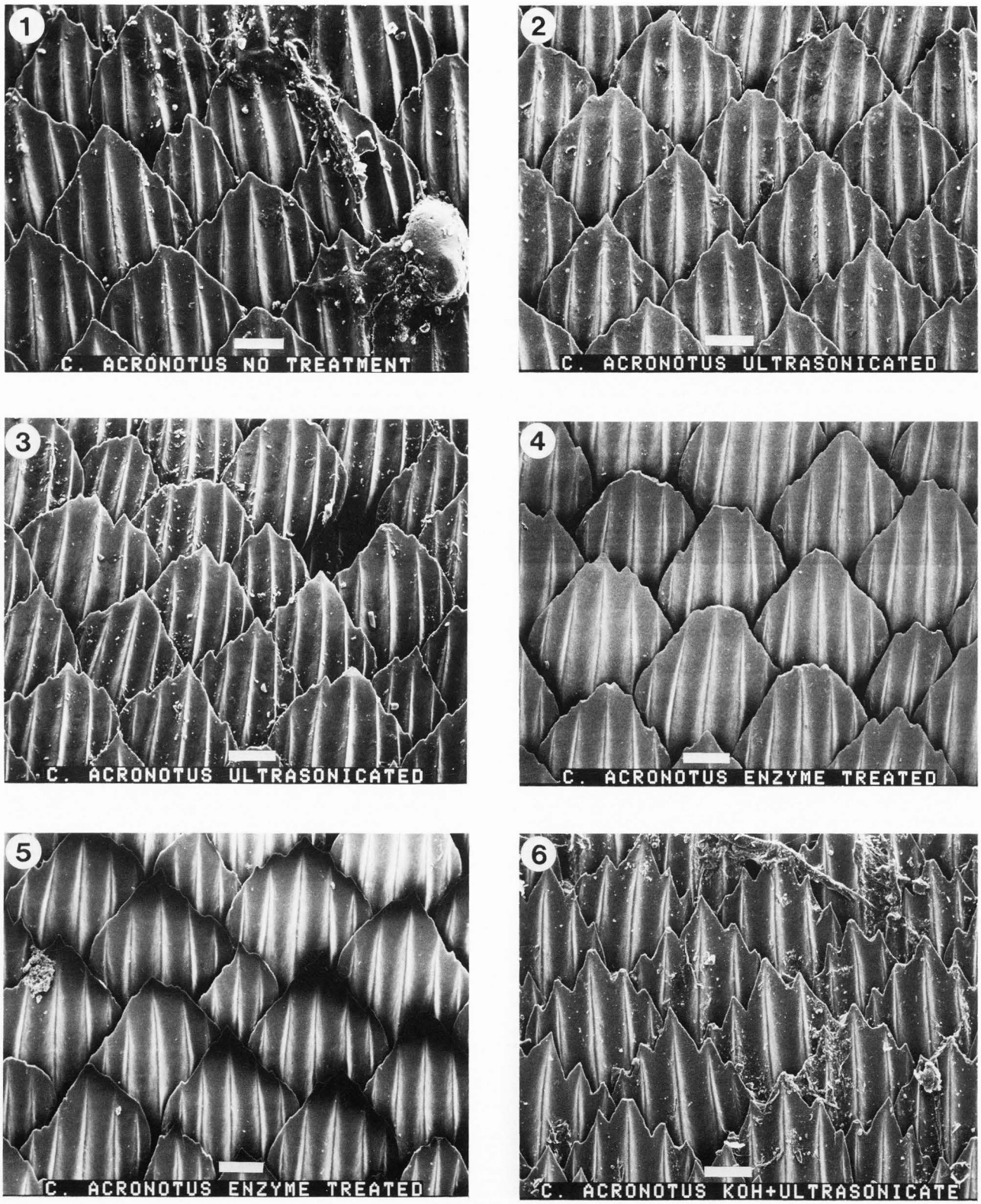
Results

Specimens with no treatment clearly show the adherence of debris to the dermal denticles (fig. 1). Cleaning with only ultrasonication, KOH, or enzyme resulted in some cleaning of debris from the denticles (figs. 2, 3, and 4 & 5, respectively) but did not yield ideal results. The enzyme cleaning had a somewhat better result than only ultrasonication or KOH. KOH followed by ultrasonication and enzyme cleaning followed by ultrasonication gave superior cleaning results than the above three treatments (figs. 6 & 7, and 8, respectively). In most cases enzyme treatment followed by ultrasonication yielded better results than KOH and ultrasonication. This can especially be seen in the tiger shark (Galeocerdo cuvieri) where on the KOH and ultrasonicated sample there are still adherences between denticles (figs. 9 & 10), whereas on the enzyme and ultrasonicated samples all such adherences have been removed. Similarly, in the sharpnose shark (Rhizopriondon porosus) the same appears to be true; the micro-relief can be seen more clearly after enzyme and ultrasonic treatment than after KOH and ultrasonic treatment (fig. 11 vs 12). However, in the great hammerhead shark (Sphyrna mokarran) the KOH and ultrasonicated samples appeared cleaner and showed the micro-relief on the denticles better than on the enzymed and ultrasonicated samples (figs. 13 & 14 vs 15 & 16, respectively). Since this micro-relief is very important in studying and analyzing shark dermal denticles, this is a critical difference. Some of this difference may be due to size of the animal. These species reach maturity at very different sizes; both specimens here are mature; the great hammerhead shark being 4 m total length, while the sharpnose shark being only 1 m. In the larger animal, the skin is much thicker and correspondingly there is more mucus. Perhaps on such thick layers the KOH works more efficaciously than the enzymes.

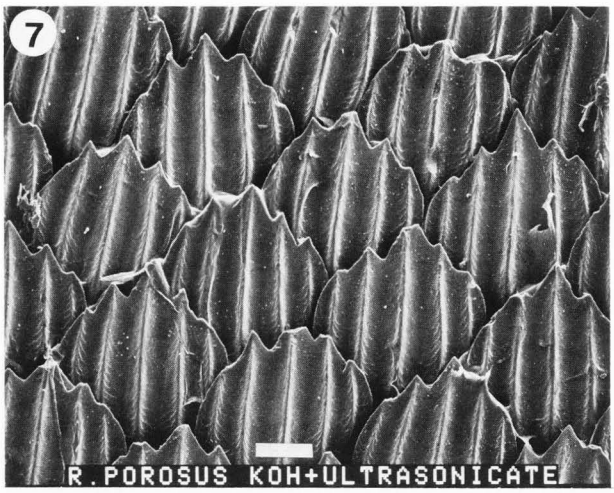
Discussion

As seen above, for most samples herein studied, an enzyme digestion followed by ultrasonication yielded the cleanest denticles for SEM observation. In a few cases, mainly larger sharks, the KOH treatment followed by ultrasonication yielded results equal to or better than the enzyme treatment. This may be due to the thicker skin, and hence more mucus, found on larger sharks. In the case of larger sharks it may also be desirable to increase the length of time they are in the KOH or enzyme and ultrasonication steps to help better clean off this thick mucus layer.

We have also used the same procedures on shark skin samples taken from preserved museum specimens with equal success. However, older specimens will often have etched dermal denticles. This is probably caused by acidification of the alcohol preservative due to the breakdown of body fats and oils into fatty acids, as noted by Dingerkus (1982). This etching will especially remove the micro-relief on the denticles. Hence,



Figs. 1-6. Blacknose shark, Carcharhinus acronotus. Anterior to bottom in each micrograph. Treatment labeled on each micrograph. Scale bars equal 100 μm. Fig. 4 taken at 10 kV, all others at 20 kV.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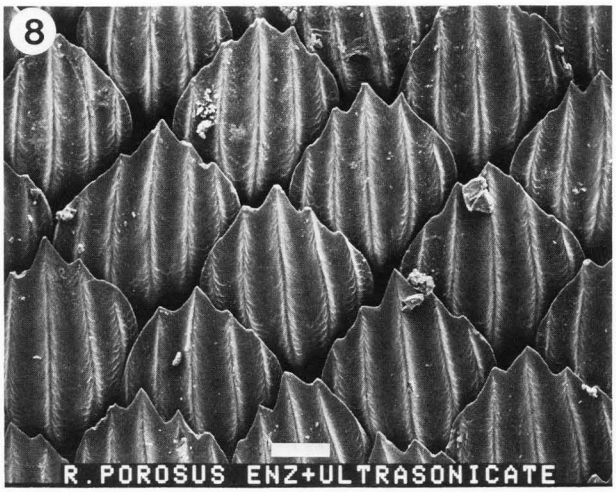
R.POROSUS KOH+ULTRASONICATE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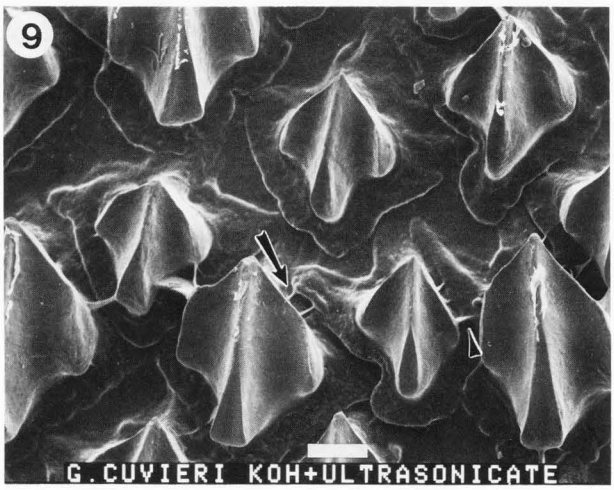
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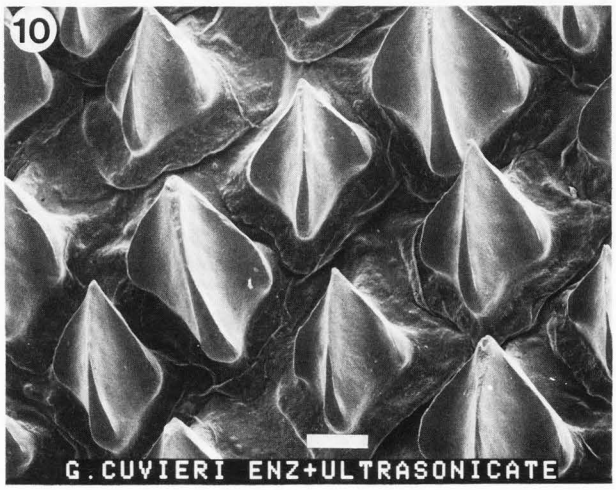
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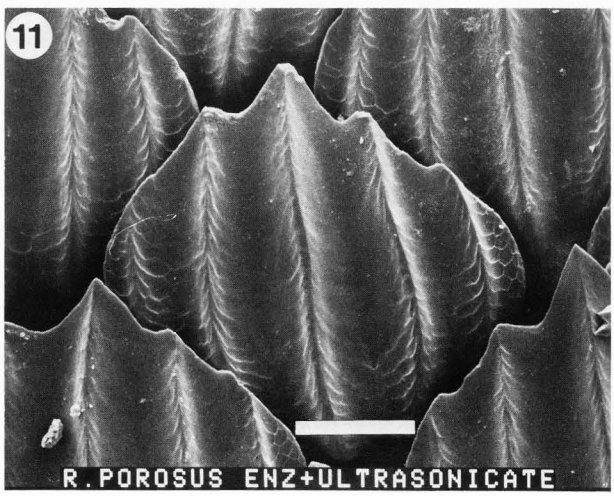
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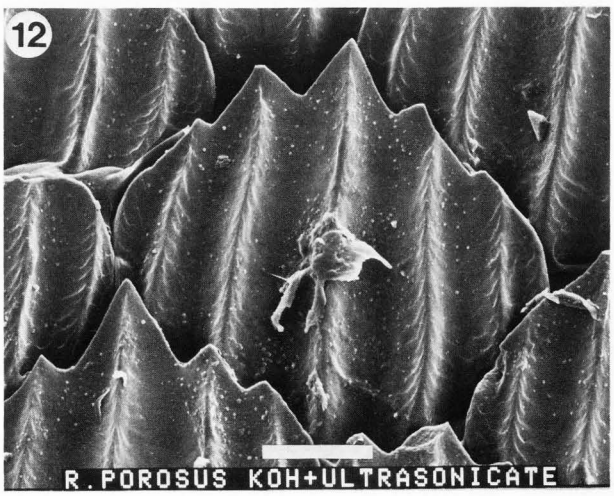
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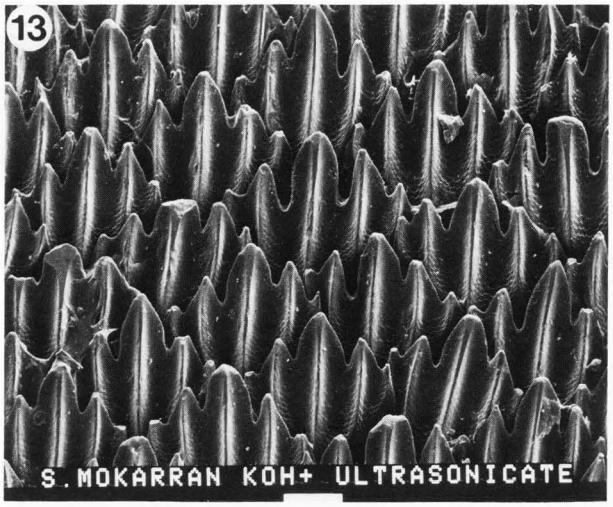
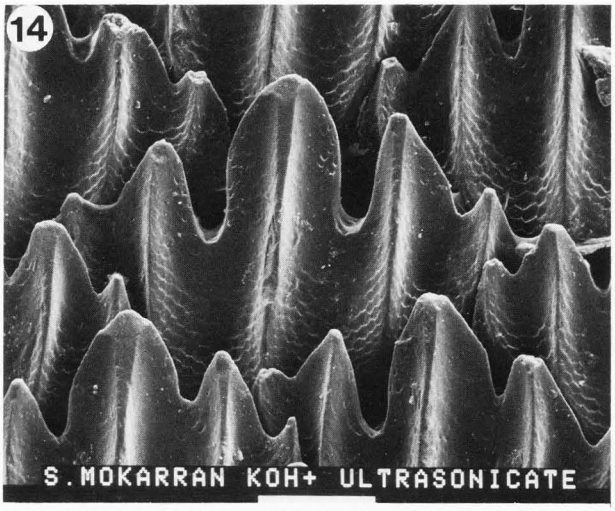
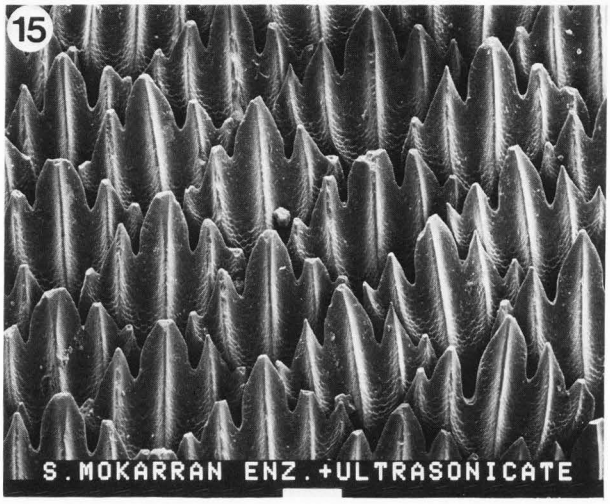
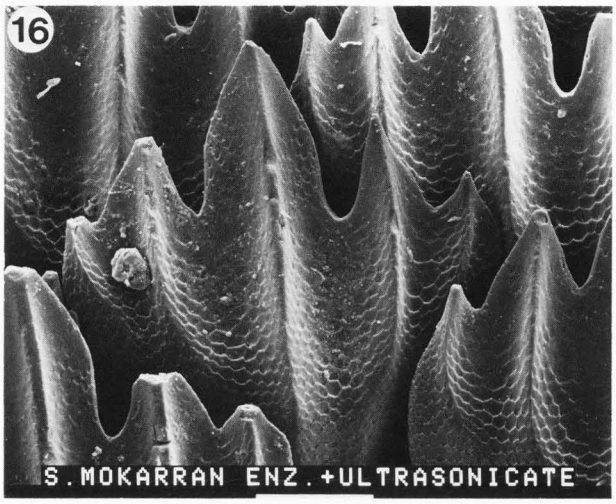






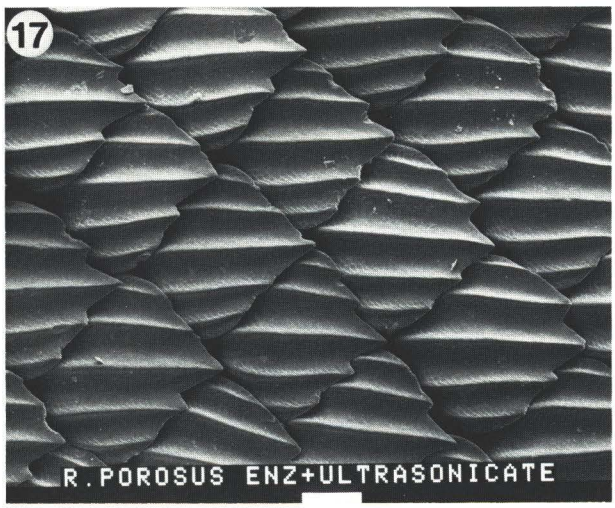


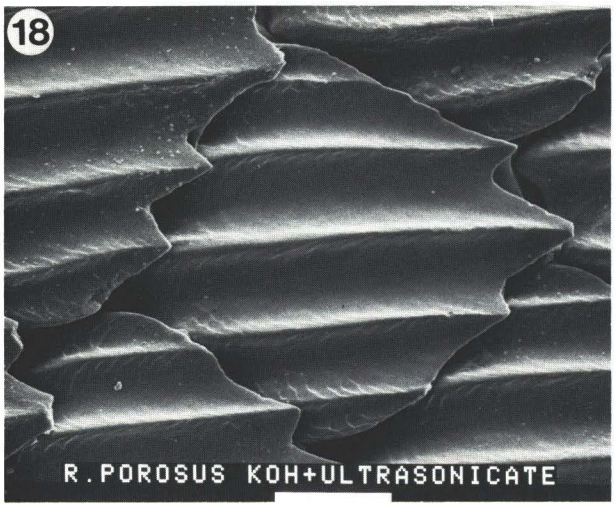
Figs. 7-8. Sharpnose shark, Rhizoprionodon porosus. Treatment labeled on each micrograph. Anterior to bottom of micrographs. Taken at 20 kV. Scale bars equal 100 μm.
Figs. 9-10. Tiger shark, Galeocerdo cuvieri. Treatment labeled on each micrograph. Anterior to bottom of micrographs. Taken at 20 kV. Scale bars equal 100 μm. On fig. 9 note adherences still present between denticles (white arrow), which are cleaned off in fig. 10.
Figs. 11-12. Sharpnose shark, Rhizoprionodon porosus. Treatment labeled on each micrograph. Anterior to bottom of micrographs. Taken at 20 kV. Scale bars equal 100 μm.

Figs. 13-16. Great hammerhead shark, Sphyrna mokarran. Anterior to bottom of each micrograph. Treatment labeled on each micrograph. Taken at 20 kV. Scale bars equal 100 μm.

for best results, it is suggested that fresh material be used in preference over preserved material wherever possible.

The coating techniques used here were modified from a 10 nm to a 15 nm gold coating during the course of this study. This was necessitated by the appearance of "charging" or distortion artifacts in the visual and micrographic images (cf. fig. 5); this need for a thicker coating also coincided with the use of more effective cleaning techniques, especially the use of enzyme treatment. The explanation for this is probably that as more dirt and adventitious material were removed from between the denticles the gold atoms had to penetrate further to achieve an effective conducting layer between denticles. While the gold sputter coating technique worked well with this series of tests, it may become necessary with other species of sharks to try sputter coating other metals, e.g., palladium, gold-palladium; or perhaps carbon coating first, with a vacuum evaporator, then sputter coating with the metal. The carbon coating technique being a straight-line deposition technique should permit a conducting layer to penetrate deeply between closely packed dermal denticles, especially when a rotating and tilting specimen stage is used.





Figs. 17-18. Sharpnose shark, Rhizoprionodon porosus. Anterior to left. Treatment labeled on each micrograph. Taken at 20 kV. Scale bars equal 100 μm.

In addition to a heavier conductive coating, which incidentally will not obscure any of the micro-relief detail, it became necessary in this study to reduce the accelerating voltage of the SEM from 20 kV to 10 kV. For example, fig. 5, taken at 20 kV, displays high contrast resulting from an uneven buildup of electrical field on the denticles. The tips of the denticles are darker because enough of the primary beam has not been conducted to ground and has caused an electrical field to buildup unevenly, this produces: (1) Suppression of the secondary electron signal (i.e., specimen electron signal that is used to produce the most common SEM image) as it tries to leave the sample surface; and (2) deflection of the primary beam before it impinges upon the surface. Reducing the operating voltage to 10 kV (fig. 4) reduces the charge build up on the surface and considerably improves the image. Although there still are charging lines (horizontal bands) apparent at 10 kV. To aid in solving this condition an additional coating of 5-10 nm of gold should be applied. The sample in figs. 4 & 5 was coated with 10 nm; subsequent samples were coated with 15 nm.

Another aspect to consider when interpreting micrographs is the orientation of the sample relative to the scanning axis of the beam. For example, compare figs. 17 & 18, with figs. 8 & 11. In both cases the beam is running horizontally from top to bottom of the picture frame. In the case of figs. 17 & 18, the denticle orientation was rotated 90° producing a shadowing effect not apparent in the other orientation. Micro-structure is more visible when the denticle is oriented perpendicular to the beam (fig. 11) than when parallel (fig. 18). It should be noted that different SEM instruments may scan vertically rather than horizontally, so care should be taken in orienting the micrographs to achieve the desired results.

In order to best study and analyze shark dermal denticles, several magnifications and orientations are usually examined and photographed. Low magnifications (usually ca. 50x) will give the overall denticle pattern, i.e., arrangement into rows, isolated, etc. Medium magnification (ca. 100x) will show how individual denticles are arranged and/or interlocked with respect to denticles around them. Slightly higher magnifications (ca. 200x) will yield the individual denticle morphology, including ridges, keels and micro-relief. Occasionally magnifications of 500-2000x will be necessary to closely examine the micro-relief and are viewed looking straight down on them. Usually denticles are arranged and photographed so that the anterior portion of the animal is oriented to the left of the viewer. However, sometimes for greater clarity or visual impact, the denticles may be oriented so that the anterior is facing the viewer. Other special views or angles (such as a 45° angle from the side) may also be used to help illustrate a special feature of the denticles, but such views must always be clearly explained.

Conclusions

As a result of these studies, we recommend that the following procedure be used as a first approach to SEM study of shark dermal denticles: 1. Skin sample is taken from the right dorso-lateral side, between the shoulder and first dorsal fin. 2. Excess flesh is scraped off the skin, and any blood or body fluids be rinsed off. 3. Skin sample is air dried in front of a fan, while being pinned out on a block of styrofoam. Once dried, skin sample is cut to desired size. 4. Enzyme digestion is performed in a solution of 30 cc saturated sodium borate (aqueous) and 70 cc distilled H_2O, and 1 g trypsin enzymes for 1 h (longer for larger sharks). 5. Ultrasonicate for 15 min. in the enzyme solution. 6. Two washings of distilled H_2O, for 15 min. each. 7. Final washing in 70% ethyl alcohol for 30 min. 8. Sample is re-dried

with a light weight on it to keep it flat. 9. Mount sample with permabond or equivalent, with light silver coating ensuring that a good conduction pathway exists between the sample and the stub. Stubs should be washed in ethyl alcohol first, then handled only with tweezers. 10. Sputter coat with 15 nm of gold. 11. Observe in the SEM at 20 kV or under.

Acknowledgements

We would especially like to thank Dr. Samuel H. Gruber, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, Florida, and to NSF Grant No. OCE 83-09831, for enabling us to participate in research cruises on the RV Cape Florida which allowed the specimen collection for this study; and Captain R. Morgan and the scientific and ship's crews of the RV Cape Florida for their assistance in the specimen collection. We would also like to thank Chris Blair for photographic assistance and Lynne Schiele for typographical assistance.

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Discussion with Reviewers

W. Raschi: While the procedures described herein will show how the denticles are arranged on the skin, wouldn't photographs of individually removed and isolated denticles [as per Raschi W, Elsam J. (1986). Comments on the structure and development of the drag reduction-type Placoid scales, in: Proceedings of the 2nd Indo-Pacific Fish Conference, Tokyo, Japan, in press.], provide an equally useful important taxonomic tool?
Authors: Yes. We have been researching the preparation techniques for isolation of individual dermal denticles, and plan a future publication on this.

J. Murphy: Which structures are used to characterize the various species?
Authors: As stated in the paper, the structures used to characterize the species are: overall shape of the denticles; ridges and keels (their size, number, and shape); and micro-relief (shape and position).

J. Murphy: If the micro-reliefs are used, should fixation and drying methods be evaluated which decrease air drying distortions? Is it possible that some of the micro-relief of the dermal tissue is caused by distortion due to surface tension upon air drying?
Authors: The dermal denticles are hard enameloid structures produced by the skin. As such the enameloid is not affected by air drying. The micro-relief is not caused by distortion, but is an integral part of the denticles as they are formed.

J. Murphy: It is stated that the purpose of this paper is to determine prep methods for high resolution SEM. This does not seem an appropriate objective since the highest magnification used is 200x.
Authors: Although the highest magnification illustrated in the present paper is 200x, higher magnifications are often necessary to study details, especially of the micro-relief. In order to study the micro-relief in some specimens we have had to use magnifications up to 2000x.

G.H. Burgess: Did you try using all three cleaning treatments--ultrasonication, enzymatic digestion, and KOH--in tandem, in addition to the pair-wise combinations reported herein? Are enzyme digestion and KOH treatments chemically incompatible if used consecutively?
Authors: The enzyme digestion and KOH treatments are not believed to be incompatible. We have not done the triple tandem procedure yet, but it is planned in further studies.

W.-E. Reif: Here is an additional article that deals in part with preparing shark skin samples for SEM: Reif, W.-E. 1985. Squamation and ecology of sharks. Courier Forschungsinstitut Senckenberg 79, Frankfurt, 255pp. with 74 plates.
Authors: Thank you for the additional reference.