Protocol for electron backscatter diffraction (EBSD) analysis of fossil eggshells

SEUNG CHOI,*[,1] YONG PARK,[1] and MIGUEL MORENO-AZANZA[2]

[1]Research Institute of Basic Sciences, Seoul National University, Seoul, 08826, South Korea; seung0521@snu.ac.kr;

[2]Aragosaurus: Recursos Geológicos y Paleoambientes, Departamento de Ciencias de la Tierra, Universidad de Zaragoza, Zaragoza, 50009, Spain

* Corresponding author

INTRODUCTION

Electron backscatter diffraction (EBSD) is an analytical technique using diffraction patterns (called Kikuchi pattern) originated from the back-scattered electrons diffracted by the lattice plane of the material which satisfies the Bragg's angle (Baba-Kishi, 2002; Kikuchi, 1928; Nishikawa & Kikuchi, 1928; Prior et al., 1999). The EBSD detector is an apparatus that attaches to either a scanning electron microscope (SEM) or transmission electron microscope (TEM). It is used by diverse fields of geology because EBSD data permit investigation of various mineralogical properties such as phase, grain size, shape, orientation, and boundary information (Prior et al., 2009). This type of analysis was initially introduced to paleontology by invertebrate paleontologists because many invertebrate fossils have mineralized hard parts that are composed of calcium carbonate ($CaCO_3$) and are relatively easy to prepare for EBSD analysis (Pérez-Huerta et al., 2007; see Cusack, 2016; Pérez-Huerta et al., 2018 and references therein).

Invertebrates are, of course, not the only taxa to use calcium carbonate as amniote vertebrates independently developed a way to synthesize calcium carbonate for reproductive purposes (Gautron et al., 2021). Among the three polymorphs of calcium carbonate (i.e., calcite, aragonite, and vaterite), calcite is most widely used for making eggshells, but turtles lay aragonitic eggshells and a few birds lay vaterite patches on the outer surface of calcitic eggshells (Board & Perrott, 1979; Hirsch, 1983). Thus, EBSD analysis already adopted by invertebrate paleontologists could easily be adopted by vertebrate paleontology to study fossil eggshells, which are probably the most numerous and widely distributed calcium carbonate fossils of vertebrates (Carpenter & Alf, 1994; Mikhailov & Zelenkov, 2020). Since the potential of EBSD analysis to study modern eggshells was introduced by Dalbeck and

Cusack (2006) and subsequently by the first paleontological study (Grellet-Tinner et al., 2011), it has become a popular technique for fossil egg research (Table 1).

EBSD mapping can be considered an advanced and complementary version of conventional histological thin section images of fossil eggshells (Quinn, 1994) because both techniques provide two-dimensional microstructural images of eggshells. Microstructure is often used for observation, identification, and classification of fossil eggshells; thus, thin sectioning has been a pivotal technique for fossil egg research since some of the earliest analyses (e.g., van Straelen, 1925). EBSD analysis has several clear advantages over the conventional technique. Firstly, EBSD is based on an electron microscope, which has a more powerful magnification capability than optical microscopy (producing micron-level resolution), so more detailed microstructural or even ultrastructural images are available. Secondly, although crystallographic information (e.g., orientation of mineral) is also available in optical microscopic images after interpreting cross-polarized light images of eggshells (e.g., Rodriguez-Navarro et al., 2002), this information is automatically presented as pseudo-colors in EBSD mappings. In other words, EBSD provides a straightforward way to understand crystallography of eggshells. Thirdly, diverse quantitative data are available in EBSD analysis (e.g., grain size, shape, boundary length, and boundary angle), whereas these are either unavailable or difficult to obtain in the conventional method. There are many more strengths associated with EBSD analysis compared with thin section imaging, so interested readers are referred to see published studies cited in Table 1.

In this contribution, we provide a detailed protocol of best practices for EBSD analysis of fossil eggshells. The steps explained in this work are also applicable to the study of modern materials such as avian (e.g., Chiang et al., 2021; Choi et al., 2023; Dalbeck & Cusack, 2006; Grellet-Tinner et al., 2017; López et al., 2023) and squamate eggshells (e.g.,

Choi et al., 2018; Deering et al., 2024; Wu et al., 2023). In the authors' experience, applying the protocols described below to modern eggshells also produces high quality results.

PROTOCOLS/METHODOLOGY

Selecting Eggshells

An eggshell fragment from any part of a fossil egg is acceptable for EBSD analysis. Nevertheless, considering that sample preparation is a destructive process because the eggshells will be embedded in non-removable epoxy resin and destroyed to expose the sections (see below), we recommend using a fragment from a discreet part of the egg. However, EBSD data acquisition can be considered non-destructive because it will produce low to no damage to the analyzed surfaces, if beam current is set properly (see below).

Any size of fragment is acceptable, but (1 cm x 1 cm) to (5 mm x 5 mm) is recommended in order to save as much of the specimen as possible. Before preparation, take a photo or micrograph to record details of the eggshell such as ornamentation, color, and pore distribution.

If the eggshell is embedded in sediments, or the pore system is infilled with sediments, it is important to consider that sedimentary and/or diagenetic infillings that are harder than calcite (Mohs scale > 3) will be difficult to polish and may produce scratches on the eggshell surface. It is therefore recommended that as much sediment as possible be removed from the eggshell prior to sectioning by means of mechanical or chemical preparation. Ultrasonic cleaning with water and hydrogen peroxide (H_2O_2) of 15% concentration is sufficient for the removal of most sediments.

Most EBSD studies are carried out on radial sections (i.e., parallel to the eggshell growth direction) as they are more informative than tangential sections (Table 1).

99

100 **Specimen Preparation**

101 Preparation of eggshells for EBSD does not differ significantly from that of other
102 carbonate specimens, but we describe it here with the intention of presenting a full procedure.
103 We highlight the precautions and strategies that are of special importance for eggshell
104 preparation. Standard EBSD analysis in a SEM requires a highly polished analytical surface.
105 This can be achieved with either ultra-polished thin sections, or ultra-polished stub samples.
106 A somewhat thicker section (> 1 mm; stub samples) can be used as an EBSD specimen, but it
107 cannot be used to get thin section images because light cannot pass through the section. A
108 thin section (0.25 to 0.60 μm) is more versatile because both EBSD data and thin section
109 images are available from the well-polished section (e.g., study of growth lines, mineralogical
110 characterization, pore density, see Moreno-Azanza et al., 2016). However, polishing the thin
111 section specimen enough for EBSD analysis can be relatively laborious and result in both
112 inappropriate preparation and specimen loss. Thus, depending on the main purpose of the
113 study, one should consider the thickness of the cut section. To facilitate specimen
114 preparation, a stub can be prepared for EBSD, and its chip counterpart can be prepared as a
115 standard thin section for further analysis, providing comparable data. Finally, although recent
116 studies have shown that EBSD data can be obtained in a TEM (see below), both in
117 transmission and reflection mode, this technique has yet to be applied to the study of fossil
118 eggshell, so here we focus on the procedure for sample preparation and EBSD data
119 acquisition in a SEM equipped with a standard EBSD detector.

120 Procedure:

121 (1) Mix epoxy resin with hardener following the manufacturer's recommended
122 proportion of epoxy and hardener. Pour the mixture (see Fig. 1A, Day 1 for appropriate
123 thickness of epoxy) into a disposable cup. We recommend a cup that is 3–4 cm in diameter.

Let the mixture consolidate (see manufacturer's manual for the recommended time for consolidation).

(2) Place eggshells on the solidified epoxy surface inside the cup. To prevent bubbles, put eggshells in a concave up arrangement. Mix epoxy resin with hardener once again and pour the mixture onto the eggshells (see Fig. 1A, Day 2 for thickness). Let the mixture consolidate.

(3) Remove the eggshells embedded in solidified epoxy. With a grinding turntable or very coarse sandpaper (e.g., 200-grit), expose the radial section of eggshells (Fig. 1A). Once the eggshells are nearly exposed, glue the flattened epoxy surface onto a glass slide. Let the glue solidify (around one day).

(4) Cut the epoxy chip to expose the radial section of the eggshell. We recommend using a cutting machine with a circular blade that can fix the glass slide (Fig. 1B). If the remaining eggshell fragment is large enough, it is useful to cut it in half and save the resulting chips as a replacement to prepare an accessory standard thin section or for curation purposes.

(5) Lap the exposed rough surface of the eggshells with abrasives (Fig. 1C). Although our explanation here is based on manual preparations with abrasive powders and their respective glass plates, sandpapers are also widely used for manual preparations. Alternatively, one can use turntables for fast and convenient lapping.

Use progressively finer abrasives to acquire a highly polished surface. Use 400-grit abrasive for one minute to erase scratches made by the circular blade. Use 600-grit abrasive for three minutes. Use 1000-grit abrasive for seven (or a few more) minutes. Use 3000-grit abrasives for 15 minutes. After this step, the surface will be smooth even under a microscope.

(6) Polish the exposed eggshells with diamond paste. Diamond grain size of either 1 μm or 0.5 μm works well (20 minutes by hand).

(7) Polish the exposed eggshells with 0.04–0.06 μm colloidal silica for 20 minutes (Fig. 1D). Check the final status of polished eggshells with reflection mode of an optical microscope (Fig. 1E). If the eggshells show very a clear reflection without any obvious scratch marks, the specimen is ready for EBSD analysis (see Pitfalls to Avoid below).

(8) Coat the prepared specimen (e.g., carbon) to make it conductive (Fig. 1F; see Pérez-Huerta & Cusack, 2009 for detailed information).

(9) Put carbon tape near the exposed eggshells and link the epoxy and glass slide to minimize the potential electrical charging effect during SEM observations and EBSD analysis (see Pitfalls to Avoid below).

Data Acquisition

(1) Attach the EBSD specimen onto a holder with carbon tapes. It should be firmly atttached because the EBSD specimen will be significantly tilted during the analysis (see below) and should not move during this time. If the analysis is expected to last several hours (i.e., large surface or old detectors), consider gluing the specimen to the holder using cyanoacrylate glue.

(2) Insert the EBSD specimen into an SEM equipped with an EBSD detector (Fig. 2A,B). Because the optimal setting for SEM and EBSD might be diverse depending on equipment, pay special attention to the EBSD model and equipment geometry. We recommend that researchers follow the recommendation of previous users or technicians in the laboratory. Published EBSD works (Table 1) usually provide their settings, so consider adopting those settings if necessary.

We recommend taking microscopic images under SEM (both secondary electron and backscattered electron images) before and after the data acquisition to track any resulting damage and to avoid loss of data.

(3) Turn on the infrared camera within the specimen chamber and tilt the stage and specimen to 70˚ (Fig. 2C). If using the pre-tilted holder, this step can be skipped. Find the best focus by adjusting the distance between the specimen and the electron gun (Fig. 2C) as well as fine-tuning the focus and stigmator functions. Be particularly careful about the distance between the specimen holder and electron gun as they must not touch each other.

(4) Insert the EBSD detector. Be careful about the distance between the specimen holder and the EBSD detector (Fig. 2C). When the detector is too far from the specimen, the detector cannot capture the Kikuchi pattern, but when they are too close to each other, the electron signal saturates the detection limit of the detector. Thus, find the appropriate distance between the detector and the specimen.

(5) At this step, we strongly recommend following the established protocols of the EBSD software manufacturers. Here, we will explain based on a software AZtec (Oxford Instruments), which is widely used.

Include the phase of calcite (most fossil eggs), aragonite (fossil turtle eggs) or any other minerals of interest. Consider adding additional calcite phases, such as high magnesium calcite (up to a 3% of Mg) that may be useful in certain specimens. Calcite, aragonite and other carbonate mineral crystal structure files are available in most EBSD pattern libraries, but they can also be constructed using data from public databases, such as the American Mineralogist Crystal Structure Database (Downs & Hall-Wallace, 2003). The software will identify the main crystallographic planes and axes by using Kikuchi pattern and index minerals based on the candidates of minerals that the researcher has included.

Make sure to capture the image of the specimen with ‘tilting corrections’ function activated. This way, one can see the corrected sectional image as if the specimen is not tilted (Fig. 2D).

Set the analysis speed and Kikuchi pattern indexing quality by selecting the analysis time and number of overlapping Kikuchi pattern images per each indexing (Fig. 2E). With increased analysis time and overlapping images, it is possible to get better Kikuchi pattern (with less false-negative pixels in EBSD maps), but it takes more time—thus, there is a compromise between the quality of results and analysis time.

Set the number of reflectors to make the threshold for Kikuchi pattern indexing (Fig. 2F). With fewer reflectors, the Kikuchi pattern indexing becomes more rigorous, but it may increase 'false-negative' in overall results. With more reflectors, the software will index more Kikuchi pattern signals, but it may contain some 'false-positive' results.

Set a targeted mapping area and step size for each pixel (Fig. 2G). The smaller a pixel is, the better the result will be. However, smaller step size increases the mapping time, so there is a tradeoff between the time and quality of results. A ratio of at least 1:10 between the step size and grain size (mostly calcite in case of eggshells) is recommended to achieve well-defined grain boundaries in EBSD analysis (Fig. 2H).

(6) At this point the acquired patterns can be stored as bitmap images or discarded. Storing patterns has the advantage of being able to re-index saved patterns with different phase minerals without the need for a new acquisition. This is of particular use in case there is uncertainty on the mineral phase composition of the eggshell (e.g., pure calcite was used, but high magnesium calcite may be better for the mineralogy of the sample). Nevertheless, keep in mind that a 1 mm^2 area of an eggshell analyzed with a 1 µm step size, will produce 10^6 patterns. Even with the best compression algorithm, stored pattern files quickly reach tens of gigabytes, which can be difficult to manipulate and curate. It is typically recommended not to store patterns unless one has a good reason to do so.

(7) Take a backscattered electron picture of the analyzer area. An identical picture should be taken once the map is finished in order to check for drift caused by beam drift or by

physical movement of the sample, in addition to possible damages in the sample. If no or minimal drift is observed between both pictures, the data is ready for the next phase.

(8) After the analysis, return the EBSD detector and specimen stage to their original places in the SEM. The EBSD detector must be moved first to prevent collision between the detector and specimen holder. Remove the EBSD specimen from the SEM.

Data Enhancement and Extraction

(1) Improve acquired EBSD maps with wild spike elimination and zero solution extrapolation (Fig. 3A). Wild spike is an inconsistent pixel surrounded by consistent background pixels. Apply wild spike elimination once. Zero solution is an unindexed pixel. It is usually caused by either a weak EBSD signal caused by a rough surface (incomplete polishing) or two overlapping Kikuchi bands in a single pixel that makes identification ambiguous. We recommend that when a zero solution is surrounded by six or more consistent pixels, it is treated as the same signal with the surrounding pixels. Apply up to three consecutive zero solution corrections. Going further than this can result in artificial growth of crystals.

(2) Extract diverse types of EBSD maps (e.g., inverse pole figure maps, Euler maps, and grain boundary maps) depending on the purpose of study (Fig. 3B; see Data Curation below).

Extract numerical data at the grain analysis tab, such as grain size, aspect ratio, and misorientation histogram (Fig. 3C).

Consider using other kinds of software for EBSD data processing (Fig. 3D–F). One can extract EBSD data of AZtec as “*.cpr/crc” or “*.ctf” file, which is universally available in other EBSD software such as MTEX (MATLAB) or HKL Channel 5 (Oxford Instruments).

247

248 **Data Curation and Interpretation**

249 The final products of these protocols are EBSD maps of eggshells, which show
250 detailed structural and crystallographic information. For the interpretation of EBSD results,
251 basic understanding for commonly-used EBSD maps is useful, so we provide an overview of
252 mapping techniques.

253 **Inverse Pole Figure Y Map**—Inverse pole figure (IPF) Y maps show the orientation
254 of the *c*-axis of calcite. A reddish color indicates that the *c*-axis lies perpendicular to eggshell
255 surface, and bluish and greenish colors indicate it is parallel to the eggshell surface (Fig. 4A).
256 The distribution of *c*-axis orientation can be summarized by {001} pole figures. The *c*-axis
257 orientations of 'reddish' calcite grains are plotted on the polar regions whereas that of
258 'bluish' and 'greenish' calcite grains are the equatorial regions (Fig. 4A). This way, the *c*-axis
259 distribution of analyzed surfaces can be compared among fossil eggshells (both intra- and
260 inter-oospecific variations).

261 **All Euler Map**—Euler maps color calcite grains based on their crystallographic
262 orientation, so similarly oriented pixels/grains share similar colors (Fig. 4B). Specifically,
263 three Euler angles φ_1, φ, and φ_2 represent the rotation about the (original) z-axis, rotated x-
264 axis, and rotated z-axis, respectively (Fig. 4B). The values of φ_1, φ, and φ_2 are assigned to
265 red, green, and blue (RGB) color gradients, respectively, and the composite color of RGB
266 gradients make the Euler color of each grain. Thus, Euler maps are useful to differentiate
267 different grains inside eggshells.

268 **Aspect Ratio Map**—Aspect ratio maps represent grain shapes of eggshells (Fig. 4C).
269 A calcite grain is mathematically approximated to an ellipse. If the ellipse is relatively round,
270 it is colored blue but if it is acute, it is colored green or even reddish colors. Shape of grains

inside fossil eggshells is variable (both intra- and inter-oospecific variations), and aspect ratio maps provide a quantitative way to show grain shapes.

Grain Boundary and Band Contrast Map—Although it is not mandatory, grain boundary and band contrast maps are often presented together because detailed grain boundary features are effectively shown above a band contrast background (Fig. 4D). Usually, grain boundary angles of 5˚–10˚, 10˚–20˚, and >20˚ are colored green, blue, and purple, respectively (note that the boundary thresholds and colors can be arbitrary selected). The grain boundary information can be summarized in misorientation histograms (Fig. 4D). The histogram is composed of neighbor-pair and random-pair angles. The angles between the two selected grains (either neighbor- or random-pair) are calculated, and then, the summed results of those numerous pairs are presented in the histogram. When the low-angle grain boundaries (i.e., green lines) are dominant in the grain boundary map, the neighbor-pair result in the 5˚–10˚ region of the histogram becomes dominant (and vice versa; see the example in Fig. 4D).

Within a single grain in band contrast maps (Fig. 4E), the region with high crystallinity yields clear Kikuchi bands, which contribute to the bright part of the map. In contrast, the region with poor crystallinity (e.g., inclusion of former organic matters, subgrain boundaries) or imperfect polishing yield obscure Kikuchi bands, which make part of the indexed grain comparatively dark. However, because intensity of Kikuchi bands is variable depending on crystallographic orientation of different grains, investigating the difference of band contrast is meaningful only inside a single grain.

See literature cited in Table 1 for more vivid examples of EBSD results of fossil eggs.

Pitfalls to Avoid

Specimen Preparation—(1) If available, use a vacuum chamber to eliminate air bubbles inside the epoxy mixture during consolidation. Bubbles near eggshells may cause electrical charging under SEM investigation and negatively affect EBSD analysis.

(2) We recommend investing sufficient time for each lapping/polishing step (the time taken may depend on experience of preparators). If the surface of the sample is inadequately lapped and polished, the final EBSD maps may show significant non-indexed regions (Fig. 5C) compared with well-prepared EBSD maps (Fig. 5A, B). In addition, pay attention to the pressure being applied to the specimen (onto the lapping/polishing plate) during preparation. If the pressure to the EBSD specimen is too low, the lapping/polishing will not be effective. In addition, some samples may be too sensitive to colloidal silica, which results in etching of the surface. Adjust this step of the polishing time accordingly.

(3) We recommend carefully cleaning the surface of the sample and glass plates using running water before moving to the next step. It is extremely important that polishing cloths and glasses are clean through the entire process, as any small particle, even coming from the specimen, may scratch the surface. If abrasive powders are not completely removed during earlier steps, they can potentially leave scratch marks on the surface of the sample. If, for example, 400-grit abrasives remain in the EBSD specimen while one laps the specimen with 3000-grit abrasives, particles of 400-grit abrasive can make scratch marks, which are hard to remove with 3000-grit abrasives. Thus, the final EBSD image may show a few scratch marks when the EBSD map is acquired (Fig. 5D).

We also recommend frequently checking for the presence of scratch marks or completeness of polishing with a reflection mode of an optical microscope (Fig. 1E). This way, significant time can be saved before running an unsuccessful EBSD analyses. Depending on the depth and width of the scratches, preparation steps 5, 6, and 7 may need to be repeated in order to remove the damage on the eggshell surface.

(4) Coat the surface of the sample with electrical conductor (such as carbon or gold) using an appropriate thickness. If the thickness of conductor is too thin or thick, a researcher may fail to get signals due to absence or weak Kikuchi bands despite using appropriate EBSD settings. When the coating is too thin, it can cause electrical charging even before the EBSD analysis. In this case, a bit more coating can resolve the problem. When the coating is too thick, Kikuchi patterns become invisible (Pérez-Huerta & Cusack, 2009). In this case, a researcher should return to the colloidal silica polishing stage and repeat step for approximately five minutes to peel off the coating. Then, recoat the EBSD specimen with an appropriate thickness.

(5) After the coating, place the carbon tapes to link the epoxy surrounding the eggshells and the glass slide to minimize the electrical charging. When it is not adequately done, the electrons do not effectively flow out from the eggshells and their surrounding epoxy, and it can cause poor EBSD images (Fig. 6).

Data Acquisition—(1) When acquiring Kikuchi pattern, a researcher may fail to get signals due to invisible Kikuchi bands under inappropriate EBSD setting. SEM equipment is sensitive and analytical conditions depend on the construction of individual instruments. In addition, EBSD detectors in different laboratories will have diverse optimal setting under respective SEM settings. Thus, when available, follow the instructions of technicians in the laboratory to find the appropriate setting for the EBSD analysis.

(2) We recommend selecting the area to be analyzed on the specimen carefully and using the weakest intensity of electron beam current/voltage possible without compromising the quality of the analysis especially when analyzing a small part of eggshell section. If the intensity of beam current/voltage is too strong and/or a small region of eggshell is mapped for a long time, the exposed polished surface may be damaged by the electron beam (Fig. 7). This means that the analyzed eggshell surface cannot be re-analyzed in future studies, which

could have negative repercussions for the reproducibility of the study as well as for any future works that should be based on the same specimen.

For thick eggshells, or when the area to be analyzed is relatively large, it may be recommendable to stitch together several overlapping maps across the eggshell thickness (e.g., Moreno-Azanza et al., 2016). This strategy will reduce the pattern distortions caused by the angle of incidence of the electron beam, and beam and/or sample drift during this time-consuming analysis. When using AZtec software, large area mapping and montage function easily allow for this step. If an EBSD map is obtained by separate duration, map-stitching can be done using ad hoc software, such as Map Stitcher, available in the Channel 5 software. New detectors have considerably reduced the acquisition time of EBSD patterns, especially in large areas, so this problem may be minimal with updated setups.

CONCLUSIONS/FUTURE DIRECTIONS

EBSD has become a useful tool in fossil egg research due to its ability to provide a wide array of quantitative and qualitative data. The advent of new detectors makes this technique fast, convenient, and relatively inexpensive. Although the initial preparation of eggshell specimens can be time consuming, following the steps described here should result in publishable EBSD data similar to those presented in the studies cited in Table 1. A new EBSD researcher may further develop her/his own innovative methodologies as well after becoming familiar with the basic protocol explained above.

Although we primarily explain the usefulness of EBSD analysis for fossil egg research, EBSD analysis appears promising for more fields of vertebrate paleontology. In our experience, fresh bone is very difficult to index due to its low crystallinity and high portion of amorphous calcium phosphate (Dorozhkin, 2010; Termine & Posner, 1967), characteristics

negative to successful EBSD analysis. However, fossil bone, which has been exposed to a degree of diagenesis (e.g. water exposure increases crystalline apatite portion in bone; Termine & Posner, 1967) may be more suitable for acquiring EBSD patterns. A few promising examples of other vertebrate tissues analyzed with EBSD can be found in the literature. For example, otoliths are also composed of calcium carbonate ($CaCO_3$; mostly aragonite). Microstructure and crystallography of modern fish otoliths were investigated with EBSD analysis (Parmentier et al., 2007; Schulz-Mirbach et al., 2013), and a recent study pioneered the analysis of fossil fish otoliths (Stolarski et al., 2023). In addition, conodont fossils are composed of bioapatite, but exposed and polished sections of conodonts have been analyzed by EBSD analysis to investigate microstructure and crystallography (Atakul-Özdemir et al., 2021; Pérez-Huerta et al., 2012). Thanks to the recent EBSD results, the controversial interpretations of conodont white matter (microcrystalline, macrocrystalline, and non-crystalline) was resolved as macrocrystalline (Atakul-Özdemir et al., 2021). This may mean that bioapatite fossils such as bones and teeth could also become the targets of EBSD analysis. In fact, even though it was done by EBSD attached to a TEM (as opposed to an SEM), one study showed that enamel of human teeth also can be analyzed (Koblischka-Veneva et al., 2018).

To summarize, although calcium carbonate is more amenable to EBSD analysis, calcium carbonates and highly crystalline bioapatite are open to this approach. Thus, it is worth trying EBSD analysis for any vertebrate fossils, which are composed of these biominerals. Although it is already a wonderful scientific tool for eggshells, EBSD has the potential to be a much more powerful tool for broader applications in vertebrate paleontology.

ACKNOWLEDGMENTS

SC thanks S. Han who shared basic knowledges for EBSD analysis, K. Tanaka for getting Japanese literature, S. Zhang for Chinese literature and information for Chinese eggshells, and X. Zhu for confirming information of Chinese eggshells EBSD data. We thank Oxford Instruments for the permission to use the screenshots of AZtec and B. Beausir for ATEX. The handling editor (Amy Balanoff), staff editor, and an anonymous reviewer greatly improved the quality and readability of the manuscript. SC was financially supported by the Leader Program of the National Research Foundation of Korea (NRF) (grant no. NRF-2020R1A3B2079815) to his principal investigator S. K. Lee; YP by Basic Science Research Program through the NRF of Korea funded by the Ministry of Education (2021R1A6A3A01087067); MMA by MCIN/AEI/10.13039/501100011033 and NextGeneration EU/PRTR (they co-finance Ramón y Cajal contract RYC2021-034473-I, PLEC2021-008203 and PID2021-122612OB-100) and the Fundação para a Ciencia e a Tecnología (PTDC/CTA-PAL/2217/2021).

AUTHOR CONTRIBUTIONS

SC designed the project and drafted the manuscript with inputs from YP and MMA. All authors edited the manuscript and confirmed the final version.

LITERATURE CITED

Atakul-Özdemir, A., Warren, X., Martin, P. G., Guizar-Sicairos, M., Holler, M., Marone, F., Martínez-Pérez, C., & Donoghue, P. C. J. (2021). X-ray nanotomography and electron backscatter diffraction demonstrate the crystalline, heterogeneous and impermeable nature of conodont white matter. *Royal Society Open Science*, *8*, 202013.

421 Baba-Kishi, K. Z. (2002). Electron backscatter Kikuchi diffraction in the scanning electron
422 microscope crystallographic analysis. *Journal of Materials Science*, *37*, 1715–1746.
423 Beausir, B., & Fundenberger, J.-J. (2017). *ATEX: Analysis tools for electron and X-ray*
424 *diffraction* (Version 3.30) [Computer software]. http://www.atex-software.eu/
425 Board, R. G., & Perrott, H. R. (1979). Vaterite, a constituent of the eggshells of the
426 nonparasitic cuckoos, *Guira guira* and *Crotophagi ani*. *Calcified Tissue International*,
427 *29*, 63–69.
428 Carpenter, K., & Alf, K. (1994). Global distribution of dinosaur eggs, nests, and babies. In K.
429 Carpenter, K. F. Hirsch, & J. R. Horner (Eds.), *Dinosaur eggs and babies* (pp. 15–30).
430 Cambridge University Press.
431 Chiang, P.-L., Tseng, Y.-C., Wu, H.-J., Tsao, S.-H., Wu, S.-P., Wang, W.-C., Hsieh, H.-I., &
432 Juang, J.-Y. (2021). Elastic moduli of avian eggshell. *Biology*, *10*, 989.
433 Choi, S., Barta, D. E., Moreno-Azanza, M., Kim, N.-H., Shaw, C. A., & Varricchio, D. J.
434 (2022). Microstructural description of the maniraptoran egg *Protoceratopsidovum*.
435 *Papers in Palaeontology*, *8*(2), e1430.
436 Choi, S., Han, S., Kim, N.-H., & Lee, Y.-N. (2018). A comparative study of eggshells of
437 Gekkota with morphological, chemical compositional and crystallographic approaches
438 and its evolutionary implications. *PLoS ONE*, *13*(6), e1099496.
439 Choi, S., Han, S., & Lee, Y.-N. (2019). Electron backscatter diffraction (EBSD) analysis of
440 maniraptoran eggshells with important implications for microstructural and taphonomic
441 interpretations. *Palaeontology*, *62*(5), 777–803.
442 Choi, S., Hauber, M. E., Legendre, L. J., Kim, N.-H., Lee, Y.-N., & Varricchio, D. J. (2023).
443 Microstructural and crystallographic evolution of palaeognath (Aves) eggshells. *eLife*,
444 *11*, e81092.

445 Choi, S., Kim, N.-H., Kim, H.-I., Kweon, J. J., Lee, S. K., Zhang, S., & Varricchio, D. J.
446 (2022). Preservation of aragonite in Late Cretaceous (Campanian) turtle eggshell.
447 *Palaeogeography, Palaeoclimatology, Palaeoecology*, *585*, 110741.
448 Choi, S., Kim, H., Paik, I., Park, Y., Jung, H., & Xu, X. (2022). Turtle eggs from the Lower
449 Cretaceous Hasandong Formation (South Korea) with relict aragonite under significant
450 thermal maturity. *Journal of Vertebrate Paleontology*, *42*(4), e2183866.
451 Choi, S., & Lee, Y.-N. (2019). Possible Late Cretaceous dromaeosaurid eggshells from South
452 Korea: A new insight into dromaeosaurid oology. *Cretaceous Research*, *103*, 104167.
453 Choi, S., Lee, S. K., Kim, N.-H., Kim, S., & Lee, Y.-N. (2020). Raman spectroscopy detects
454 amorphous carbon in an enigmatic egg from the Upper Cretaceous Wido Volcanics of
455 South Korea. *Frontiers in Earth Science*, *7*, 349.
456 Choi, S., Moreno-Azanza, M., Csiki-Sava, Z., Prondvai, E., & Lee, Y.-N. (2020).
457 Comparative crystallography suggests maniraptoran theropod affinities for latest
458 Cretaceous European 'geckoid' eggshell. *Papers in Palaeontology*, *6*(2), 265–292.
459 Choi, S., Park, Y., Kweon, J. J., Kim, S., Jung, H., Lee, S. K., & Lee, Y.-N. (2021). Fossil
460 eggshells of amniotes as a paleothermometry tool. *Palaeogeography, Palaeoclimatology,*
461 *Palaeoecology*, *571*, 110376.
462 Cusack, M. (2016). Biomineral electron backscatter diffraction for palaeontology.
463 *Palaeontology*, *59*(2), 171–179.
464 Dalbeck P., & Cusack, M. (2006). Crystallography (electron backscatter diffraction) and
465 chemistry (electron probe microanalysis) of the avian eggshell. *Crystal Growth &*
466 *Design*, *6*(11), 2558–2562.
467 Deering, J., Nelea, V., & McKee, M. D. (2024). Multiscale mineralization in the leopard
468 gecko eggshell. *Advanced Functional Materials*, 2316422.

469 Demarchi, B., Stiller, J., Grealy, A., Mackie, M., Deng, Y., Gilbert, T., Clarke, J., Legendre,
470 L. J., Boano, R., Sicheritz-Pontén, T., Magee, J., Zhang, G., Bunce, M., Collins, M. J., &
471 Miller, G. (2022). Ancient proteins resolve controversy over the identity of *Genyornis*
472 eggshell. *Proceedings of the National Academy of Sciences of the United States of*
473 *America*, *119*(43), e2109326119.
474 Dorozhkin, S. V. (2010). Amorphous calcium (ortho)phosphates. *Acta Biomaterialia*, *6*,
475 4457–4475.
476 Downs, R. T., & Hall-Wallace, M. (2003). The *American Mineralogist* crystal structure
477 database. *American Mineralogist*, *88*(1), 247–250.
478 Eagle, R. A., Enriquez, M., Grellet-Tinner, G., Pérez-Huerta, A., Hu, D., Tütken, T.,
479 Montanari, S., Loyd, S. J., Ramirez, P., Tripati, A. K., Kohn, M. J., Cerling, T. E.,
480 Chiappe, L. M., & Eiler, J. M. (2015). Isotopic ordering in eggshells reflects body
481 temperatures and suggests differing thermophysiology in two Cretaceous dinosaurs.
482 *Nature Communications*, *6*, 8296.
483 Ferrill, D. A., Morris, A. P., Evans, M. A., Burkhard, M., Groshong R. H., Jr., & Onasch, C.
484 M. (2004). Calcite twin morphology: A low-temperature deformation geothermometer.
485 *Journal of Structural Geology*, *26*, 1521–1529.
486 Gautron, J., Stapane, L., Le Roy, N., Nys, Y., Rodriguez-Navarro, A. B., & Hincke, M. T.
487 (2021). Avian eggshell biomineralization: An update on its structure, mineralogy and
488 protein tool kit. *BMC Molecular and Cell Biology*, *22*, 11.
489 Grellet-Tinner, G., Lindsay, S., & Thompson, M. B. (2017). The biomechanical, chemical
490 and physiological adaptations of the eggs of two Australian megapodes to their nesting
491 strategies and their implications for extinct titanosaur dinosaurs. *Journal of Microscopy*,
492 *267*(2), 237–249.

493 Grellet-Tinner, G., Murelaga, X., Larrasoaña, J. C., Silveira, L. F., Olivares, M., Ortega, L.
494 A., Trimby, P. W., & Pascual, A. (2012). The first occurrence in the fossil record of an
495 aquatic avian twig-nest with Phoenicopteriformes eggs: Evolutionary implications. *PLoS*
496 *ONE*, *7*(10), e46972.
497 Grellet-Tinner, G., Sim, C. M., Kim, D. H., Trimby, P., Higa, A., An, S. L., Oh, H. S., Kim,
498 T., & Kardjilov, N. (2011). Description of the first lithostrotian titanosaur embryo *in ovo*
499 with Neutron characterization and implications for lithostrotian Aptian migration and
500 dispersion. *Gondwana Research*, *20*, 621–629.
501 Grellet-Tinner, G., Spooner, N. A., & Worthy, T. H. (2016). Is the "Genyornis" egg of a
502 mihirung or another extinct bird from the Australian dreamtime? *Quaternary Science*
503 *Reviews*, *133*, 147–164.
504 Han, F., Yu, Y., Zhang, S., Zeng, R., Wang, X., Cai, H., Wu, T., Wen, Y., Cai, S., Li, C., Wu,
505 R., Zhao, Q., & Xu, X. (2024). Exceptional Early Jurassic fossils with leathery eggs shed
506 light on dinosaur reproductive biology. *National Science Review*, *11*(6), nwad258.
507 He, Q., Zhang, S., Xing, L., Jiang, Q., Wang, X., Pan, Z., & Hu, Y. (2019). A new oospecies
508 of Similifaveoloolithidae from the Xiuning Basin, Late Cretaceous of Anhui, China.
509 *Historical Biology*, *31*(2), 168–176.
510 Hirsch, K. F. (1983). Contemporary and fossil chelonian eggshells. *Copeia*, *1983*(2), 382–
511 397.
512 Hirsch, K. F., & Quinn, B. (1990). Eggs and eggshell fragments from the Upper Cretaceous
513 Two Medicine Formation of Montana. *Journal of Vertebrate Paleontology*, *10*(4), 491–
514 511.
515 Jain, S., Bajpai, S., Kumar, G., & Pruthi, V. (2016). Microstructure, crystallography and
516 diagenetic alteration in fossil ostrich eggshells from Upper Palaeolithic sites of Indian
517 peninsular region. *Micron*, *84*, 72–78.

518 Kikuchi, S. (1928). Diffraction of cathode rays by mica. *Japanese Journal of Physics*,
519 *5*(3061), 83–96.
520 Kim, N.-H., Choi, S., Kim, S., & Lee, Y.-N. (2019). A new faveoloolithid oogenus from the
521 Wido Volcanics (Upper Cretaceous), South Korea and a new insight into the oofamily
522 Faveoloolithidae. *Cretaceous Research*, *100*, 145–163.
523 Koblischka-Veneva, A., Koblischka, M. R., Schmauch, J., & Hannig, M. (2018). Human
524 dental enamel: A natural nanotechnology masterpiece investigated by TEM and t-EBSD.
525 *Nano Research*, *11*(7), 3911–3921.
526 Kundrát, M., & Cruickshank, A. R. I. (2022). New information on multispherulitic dinosaur
527 eggs: Faveoloolithidae and Dendroolithidae. *Historical Biology*, *34*(6), 1072–1084.
528 López, A. V., Choi, S., Park, Y., Hanley, D., Lee, J.-W., Honza, M., & Bolmaro, R. E.
529 (2023). Avian obligate brood parasitic lineages evolved variable complex polycrystalline
530 structures to build tougher eggshells. *iScience*, *26*(12), 108552.
531 Mikhailov, K. E. (1997). *Avian eggshells: An atlas of scanning electron micrographs*. British
532 Ornithologists Club Occasional Publications.
533 Mikhailov, K. E., & Zelenkov, N. (2020). The late Cenozoic history of the ostriches (Aves:
534 Struthionidae), as revealed by fossil eggshell and bone remains. *Earth-Science Reviews*,
535 *208*, 103270.
536 Moreno-Azanza, M., Bauluz, B., Canudo, J. I., Gasca, J. M., & Torcida Fernández-Baldor, F.
537 (2016). Combined use of electron and light microscopy techniques reveals false
538 secondary shell units in Megaloolithidae eggshells. *PLoS ONE*, *11*(5), e0153026.
539 Moreno-Azanza, M., Bauluz, B., Canudo, J. I., & Mateus, O. (2017). The conservative
540 structure of the ornithopod eggshell: Electron backscatter diffraction characterization of
541 *Guegoolithus turolensis* from the Early Cretaceous of Spain. *Journal of Iberian Geology*,
542 *43*, 235–243.

543 Moreno-Azanza, M., Díaz-Berenguer, E., Silva-Casal, R., Pérez-García, A., Badiola, A., &
544 Canudo, J. I. (2021). Recognizing a lost nesting ground: First unambiguous Testudines
545 eggshells from the Eocene, associated with the pleurodiran *Eocenochelus* (Huesca,
546 Northern Spain). *Palaeogeography, Palaeoclimatology, Palaeoecology*, *576*, 110526.
547 Moreno-Azanza, M., Mariani, E., Bauluz, B., & Canudo, J. I. (2013). Growth mechanisms in
548 dinosaur eggshells: An insight from electron backscatter diffraction. *Journal of*
549 *Vertebrate Paleontology*, *33*(1), 121–130.
550 Nishikawa, S., & Kikuchi, S. (1928). Diffraction of cathode rays by calcite. *Nature*,
551 *122*(3080), 726.
552 Oser, S. E., Chin, K., Sertich, J. J. W., Varricchio, D. J., Choi, S., & Rifkin, J. (2021). Tiny,
553 ornamented eggs and eggshell from the Upper Cretaceous of Utah represent a new
554 ootaxon with theropod affinity. *Scientific Reports*, *11*, 10021.
555 Parmentier, E., Cloots, R., Warin, R., & Henrist, C. (2007). Otolith crystals (in Carapidae):
556 Growth and habit. *Journal of Structural Biology*, *159*, 462–473.
557 Pérez-Huerta, A., & Cusack, M. (2009). Optimizing electron backscatter diffraction of
558 carbonate biominerals—Resin type and carbon coating. *Microscopy and Microanalysis*,
559 *15*, 197–203.
560 Pérez-Huerta, A., Coronado, I., & Hegna, T. A. (2018). Understanding biomineralization in
561 the fossil record. *Earth-Science Reviews*, *179*, 95–122.
562 Pérez-Huerta, A., Cusack, M., & England, J. (2007). Crystallography and diagenesis in fossil
563 craniid brachiopods. *Palaeontology*, *50*(4), 757–763.
564 Pérez-Huerta, A., Cusack, M., & Méndez, C. A. (2012). Preliminary assessment of the use of
565 electron backscatter diffraction (EBSD) in conodonts. *Lethaia*, *45*(2), 253–258.
566 Prior, D. J., Boyle, A. P., Brenker, F., Cheadle, M. C., Day, A., Lopez, G., Peruzzo, L., Potts,
567 G. J., Reddy, S., Spiess, R., Timms, N. E., Trimby, P., Wheeler, J., & Zetterström, L.

(1999). The application of electron backscatter diffraction and orientation contrast imaging in the SEM to textural problems in rocks. *American Mineralogists*, *84*, 1741–1759.

Prior, D. J., Mariani, E., & Wheeler, J. (2009). EBSD in the Earth sciences: Applications, common practice, and challenges. In A. J. Schwartz, M. Kumar, B. L. Adams, & D. P. Field (Eds.), *Electron backscatter diffraction in materials science* (pp. 345–360). Springer.

Quinn, B. (1994). Fossilized eggshell preparation. In P. Leiggi & P. May (Eds.), *Vertebrate paleontological techniques, vol. 1* (pp. 146–153). Cambridge University Press.

Rodriguez-Navarro, A., Kalin, O., Nys, Y., & Garcia-Ruiz, J. M. (2002). Influence of the microstructure on the shell strength of eggs laid by hens of different ages. *British Poultry Science*, *43*(3), 395–403.

Schulz-Mirbach, T., Götz, A., Griesshaber, E., Plath, M., & Schmahl, W. W. (2013). Texture and nano-scale internal microstructure of otoliths in the Atlantic molly, *Poecilia mexicana*: A high-resolution EBSD study. *Micron*, *51*, 60–69.

Stolarski, J., Drake, J., Coronado, I., Vieira, A. R., Radwańska, U., Heath-Heckman E. A. C., Mazur, M., Guo, J., & Meibom, A. (2023). First paleoproteome study of fossil fish otoliths and the pristine preservation of the biomineral crystal host. *Scientific Reports*, *13*, 3822.

Termine, J. D., & Posner, A. S. (1967). Amorphous/crystalline interrelationships in bone mineral. *Calcified Tissue Research*, *1*, 8–23.

Uematsu, R., Tanaka, K., Kozu, S., Isaji, S., & Shimojima, S. (2023). Fossil eggshells from the Early Cretaceous Okurodani Formation, northern central Japan. *Historical Biology*, *35*(12), 2396–2407.

592 Van Straelen, V. (1925). The microstructure of the dinosaurian egg-shells from the
593 Cretaceous beds of Mongolia. *American Museum Novitates*, *173*, 1–4.
594 Wu, H.-J., Tseng, Y.-C., Tsao, S.-H., Chiang, P.-L., Tai, W.-Y., Hsieh, H.-I., Yu, H.-T., &
595 Juang, J.-Y. (2023). A comparative study on the microstructures, mineral content, and
596 mechanical properties of non-avian reptilian eggshells. *Biology*, *12*, 688.
597 Wu, R., Niu, K., Zhang, S., Xue, Y., & Han, F. (in press). A new ootype of putative
598 dromaeosaurid eggs from the Upper Cretaceous of southern China. *Cretaceous Research*.
599 https://doi.org/10.1016/j.cretres.2024.105909
600 Xu, L., Xie, J., Zhang, S., Choi, S., Kim, N.-H., Gao, D., Jin, X., Jia, S., & Gao, Y. (2022).
601 Fossil turtle eggs from the Upper Cretaceous Gaogou Formation, Xiaguan-Gaoqiu Basin,
602 Neixiang County, Henan Province, China: Interpretation of the transformation from
603 aragonite to calcite in fossil turtle eggshell. *Cretaceous Research*, *134*, 105166.
604 Zhu, X., Wang, Q., & Wang, X. (in press). Electron backscatter diffraction (EBSD) study of
605 elongatoolithid eggs from China with microstructural and parataxonomic implications.
606 *Paleobiology*. https://doi.org/10.1017/pab.2024.9
607

608 Submitted February 28, 2024; revisions received Month DD, YYYY; accepted Month DD,
609 YYYY. Handling Editor: Amy Balanoff

610 FIGURE CAPTIONS

611

612 FIGURE 1. EBSD specimen preparation. **A**, a good example of exposed eggshells embedded
613 in epoxy resin. Note the appropriate thickness of epoxy resin surrounding the eggshells (~1
614 cm total) and the size of cup (~3 cm); **B**, cutting process with a circular blade; **C**, lapping
615 process with abrasive powders on a glass plate; **D**, polishing by colloidal silica; **E**,

occasionally check the quality of lapping/polishing with reflection mode of a light microscope; **F**, carbon coating. [planned for 2/3 page width]

FIGURE 2. Data acquisition by EBSD analysis. **A**, inserting EBSD specimen (circle) into the SEM; **B**, EBSD detector (arrow) attached to the SEM; **C**, inside view of the specimen chamber of SEM. Note that EBSD specimen holder is tilted at 70˚ to the normal direction of the screen of detector to get strongest diffracted electron signal, which is a key factor of EBSD analysis. The dashed arrows mark the projection of electron beam; **D–H**, step-by-step EBSD analysis by AZtec (v. 4.3; Oxford Instruments). The arrows in E and F mark the high-quality Kikuchi bands and their automatic indexing. [planned for page width]

FIGURE 3. EBSD data enhancement and extraction. **A**, wild spike elimination and zero solution correction; **B**, extracting diverse EBSD maps; **C**, extracting grain measurements and other numerical variables; **D**, screenshot of ATEX (Beausir & Fundenberger, 2017); **E**, PFch5; **F**, MTEX. [planned for page width]

FIGURE 4. Examples of expected outcomes (based on an eggshell of the Cenozoic paleoganth *Lithornis*; Table 1). **A**, an inverse pole figure (IPF) Y map and pole figures. The dominant reddish grains (e.g., α, β, and γ) contribute to the polar regions of the {001} pole figure, but the minor bluish grains (e.g., δ) contribute to the equatorial region. The polar regions show the stronger signal. The color bar shows the range of multiple of uniform density (MUD). **B**, an all-Euler map. In the right image, the three points x, y, and z move to the x′, y′, and z′, respectively, after doing φ_1 (red arrow), φ (green), and φ_2 (blue) rotations in order. **C**, an aspect ratio map. **D**, a grain boundary map overlying a band contrast map, misorientation histogram, and a schematic figure of two types of misorientations. A

misorientation histogram shows the distribution of both neighbor-pair (blue) and random pair (red) misorientations in the eggshell. The numbers in the x- and y-axis of the histogram indicate frequency and angle (degree), respectively. **E**, an example of different band contrasts. A black scale bar equals 250 μm (A) and all whole-eggshell-size EBSD maps (A–D) share the same scale. [planned for page width]

FIGURE 5. Successful and unsuccessful examples of EBSD analysis. **A,** a good EBSD map of fossil (*Lithornis*) eggshells. The hatch-mark structure (arrows) is calcite twinning, which is called herringbone pattern in some literature (e.g., He et al., 2019:fig. 4A, B; Hirsch & Quinn, 1990:fig. 10F, 11E; Uematsu et al., 2023:fig. 2B). It is caused by diagenetic processes (Choi et al., 2021; Ferrill et al., 2004) common in fossil eggs but not present in modern eggshells; **B**, a good EBDS map of modern bird eggshell (cuckoo). There are many empty spaces (arrows), but it is a biogenic feature of most modern avian eggshells (see Mikhailov, 1997); **C**, a poorly prepared modern avian eggshell. If the lapping and polishing are not adequate, there can be poorly indexed regions, which are characterized by significant area of zero solutions (arrows); **D**, scratch marks. If abrasives of earlier lapping steps (with coarse abrasives) remain during the next step (with fine abrasives), the coarse abrasives can make scratch marks (arrows). Scale bars equal 250 μm (A); 50 μm (B–D). [planned for 2/3 page width]

FIGURE 6. An example of electrical charging. **A**, due to the lack of carbon tapes that link the epoxy and glass slide, there exist significant electrical charging that spoils the quality of the image; **B**, the same region of eggshell after putting carbon tapes and making linkage between the two parts. Scale bars equal 50 μm (A, B). [planned for column width]

FIGURE 7. An example of a damaged map. **A**, full image of the eggshell. Two subsequent analyses were done at regions B and C (in order); **B**, a small region was analyzed; **C**, after analyzing B, one more map was acquired at C. Due to the damage caused by the strong electron beam applied to the small area in B, the damaged region made the specimen unsuitable for further analysis (arrow). Scale bars equal 50 μm (A); 25 μm (C); 10 μm (B). [planned for page width]