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Microstructure, crystallography and diagenetic alteration in fossil ostrich eggshells from Upper Palaeolithic sites of Indian peninsular region

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ABSTRACT

Biominerals studies are of importance as they provide an understanding of natural evolutionary processes. In this study we have investigated the fossil ostrich eggshells using Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD) and Electron Backscatter Diffraction (EBSD). SEM studies demonstrated the ultrastructure of fossil eggshells and formation of calcified cuticular layer. The presence of calcified cuticle layer in eggshell is the basis for ancient DNA studies as it contains preserved biomolecules.

EBSD accentuates the crystallographic structure of the ostrich eggshells with sub-micrometer resolution. It is a non-destructive tool for evaluating the extent of diagenesis in a biomineral. EBSD analysis revealed the presence of dolomite in the eggshells. This research resulted in the complete recognition of the structure of ostrich eggshells as well as the nature and extent of diagenesis in these eggshells which is vital for genetic and paleoenvironmental studies.

1. Introduction

Ratite eggshells are reported from more than forty Upper Paleolithic sites in peninsular India (Kumar et al., 1988). These fossil eggshells were dated from 25,000 to 40,000 years BP, either using radiocarbon dates or the presence of Upper Paleolithic tools (Kumar et al., 1988, 1990). Fossil eggshell fragments are biomineralized skeletal remains that provide important taxonomic, functional morphology, and evolutionary data (Trimby and Grellet-Tinner, 2011). For example, fossilized eggshells, which are considered to be of Godwanan origin, confirm the presence of ostriches in central and southern Asia, Africa, Middle East, and Europe in prehistoric times, whereas the more reduced geographical distribution of ratites nowadays is possibly related to an aftereffect of the continental drift (Cooper et al., 2001; Cracraft, 1974; Sibley and Ahlquist, 1990a).

Fossil eggshells rarely encompass embryonic remains either due to cracking of eggs during burial process or being damaged by microbial activity. Therefore, paleontologists rely on morphological aspects to study their origin and phylogenetic relationships prehistoric avian species. Also, the study of morphology, composition and microstructure is essential to understand the formation of avian eggshell (Richards et al., 2000). The process of formation of the eggshell in avian species is extremely rapid (Feng et al., 2001) and it has a characteristic and conservative structure consisting of several layers (mammillary layer, palisade layer, and cuticle layer) from the inner surface to the outer surface (Fig. 1). Indian fossil eggshells have the closest similarity with the widely distributed Neogene fossil taxon Struthiolithus, on the basis of combination of eggshell surface, pore, pore canal morphology and cross-sectional features (Patnaik et al., 2009). Ratites monophyly is extensively recognized on the basis of morphological, skeletal (Cracraft, 1974; Lee et al., 1997), and oological studies (Cooper et al., 2001; Sibley and Ahlquist, 1990b). However, these studies are not conclusive about the phylogenetic relationships and origin of fossilized eggshells from India. DNA based species identification of these eggshells is necessary to understand the phylogeny of these ostriches. Prior to DNA analyses, the preservation of these fossils have to be determined, even if they appear well preserved on the basis of external morphology.

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Table 1
Sample collection sites and their respective states.

Sl no.	Site	Area	State & GPS co-ordinates	Catalogue no.
1	Chavni Baroda	Bundi	Rajasthan (25° 26′ 24″ N, 75° 38′ 24″ E)	SJ/CB/001
2	Anjar	Kachchh	Gujarat (23° 6′ 48.32″ N, 70° 1′ 39.88″ E)	SB/AN/003
3	Chandresal-1	Kota	Rajasthan (25° 10′ 48″ N, 75° 49′ 48″ E)	GK/CH1/004
4	Chandresal-2	Kota	Rajasthan (25°13′ 34.1″ N 75°55′ 34.7″ E)	GK/CH2/006
5	Nagda	Chambal	Rajasthan (25°10′ 53.3″ N 75°48′ 45.5″ E)	GK/NA/008

The aim of this study is to determine the degree of preservation of Indian fossil eggshells of ostriches using a combination of Scanning Electron Microscopy (SEM), and X-ray Diffraction (XRD), Electron Backscatter Diffraction (EBSD), and X-ray Diffraction (XRD). SEM studies shed light on the formation of the different layers of eggshells and how the cuticular layers preserves biomineral structures enriched in organic matrix, mainly composed of proteoglycans and glycoproteins, from which genetic information can be extracted. XRD data is informative of the eggshell mineralogy and the degree of mineral replacement associated to diagenesis. Finally, EBSD has emerged as a high-resolution, in situ technique for providing crystallographic information of biominerals (e.g., Dalbeck et al., 2006; Dalbeck and Cusack, 2006; Cusack et al., 2007; England et al., 2007; Perez-Huerta et al., 2007; Pérez-Huerta et al., 2011) and to detect diagenesis (e.g., Cusack et al., 2008). It has been recently applied on studying eggshell structures (Trimby and Grellet- Tinner, 2011) and for palaeontology (Cusack, 2015).

This study would result in the complete crystallographic structure of ostrich eggshells as well as extent of preservation of biominerals in the eggshells, which is an essential requirement for genetic, paleoenvironmental and paleodietary studies.

2. Materials and methods

2.1. Materials

Fossil eggshell fragments were recovered from five different localities of India as shown in Table 1, and one modern African ostrich eggshell was also analyzed as control sample. The fossil eggshells collected from Indian sites were dated from 25,000 to 40,000 years BP, either using radiocarbon dates or the presence of Upper Paleolithic tools (Kumar et al., 1988, 1990).

2.2. Methods

2.2.1. Scanning Electron Microscopy (SEM)

Naural surfaces were prepared for SEM observations. Fragments of ostrich eggshells were mounted on aluminium stubs so that the outer surface (attached to the mammillary layer) face the detector. The eggshells were coated (8–10 nm) with gold sputtering (Richards et al., 2000) using BALTEC SCD005 for 40–50 s. They were then observed under a FEI Quanta 200 F.

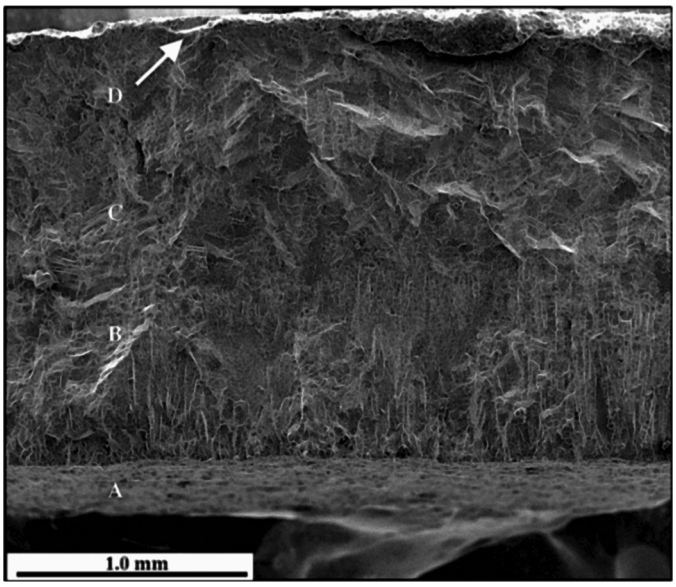

Fig. 2. SEM morphology of the cross-sectional plane of ostrich eggshell (SB/AN/003). (A) Organic membrane (B) cone layer (C) palisade layer(D) cuticle layer.

2.2.2. X-ray Diffraction (XRD)

XRD data were recorded with CuKα radiation over the 2 h range from 5 to 100 with the scanning rate of 2 $min^{-1}\theta$ (Feng et al., 2001). Plaque and powdered samples were analyzed to reveal the mineralogy of different layers. Origin software was used to analyze the plaque samples data and JADE is used to plot the powdered XRD data.

2.2.3. Electron Backscatter Diffraction (EBSD)

Two fossil eggshells, from Bundi and Chandresal, and one modern ostrich eggshell was chosen for the EBSD analysis. Eggshell fragments were embedded in epoxy resin to observe cross sections, from outer to inner surfaces, throughout the shell thickness. Samples were grinded and subsequently polished with alumina of 1 μm and 0.3 μm and finally with colloidal silica (0.06 μm). Before analysis, samples were coated with a thin layer (2.5 nm) of carbon (Pérez-Huerta and Cusack, 2009) and the samples were surrounded by silver paint to avoid electron charging. The EBSD study was carried out with an Oxford Nordlys camera mounted on a Field Emission Scanning Electron Microscope (FE-SEM) JEOL 7000 located in the Central Analytical Facility (CAF) of The University of Alabama. EBSD data were collected with Oxford Aztec 2.0 soft-

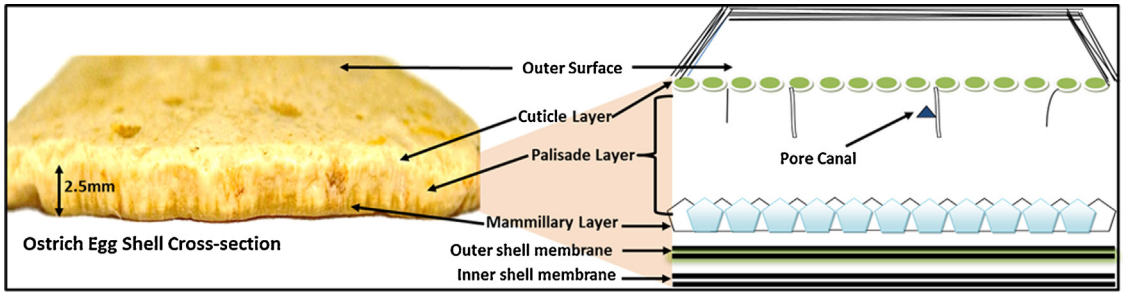

Fig. 1. Image of an ostrich eggshell radial cross-section. Image with a corresponding schematic representation showing the cuticle layer, the palisade layer, and the mammillary layer of the eggshell.