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Deep echinoderm phylogeny preserved in organic molecules from Paleozoic fossils

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INTRODUCTION

The presence of fossilized organic molecules has been documented with various geochemical and imaging technology, which is both a strength and a perceived problem for this field. First, the fact that detection of taxon-specific organic molecules is, in general, technique independent is a strength. Alternatively, varying techniques between labs has raised questions (Wolkenstein, 2014; O'Malley, 2014), which needlessly add doubt to the basic result of the relatively common existence of fossilized organic molecules in echinoderms. Extracts are the geologically stable products of original organic molecules in most, if not all, instances. This raises new, interesting questions and is a fruitful area of future research, but it does not detract from the fact that taxon-specific organic molecules can be extracted directly from individual Mesozoic and Paleozoic fossils. The present paper is a contribution building on previous research of preserved organic molecules in Paleozoic echinoderms.

ECHINODERM MORPHOLOGICAL DISPARITY

Because of their multi-plated, mesodermal skeletons, the morphological disparity of echinoderms is very high. Symmetries include bilateral, pentaradiate, radial, helically twisted, and irregular. Adult body size varies from less than 2 mm to crinoids with stems in excess of 20 m in height (Hess et al., 1999). Skeletons vary from firmly cemented plates to flexible plating to the absence of plating. Consequently, much effort has been expended to document and understand morphological disparity both among echinoderm classes and within echinoderm clades (Foote, 1992, 1994; Sumrall and Wray, 2007; Villier and Eble, 2004; Lefebvre et al., 2006; Deline and Ausich, 2011; Zamora et al., 2012; Deline, 2015). Morphological disparity between clades may be sufficiently high that virtually no discrete homologous characters can be identified other than synapomorphies for the Echinodermata as a whole (e.g., compare helicoplacoids to blastoids). This lack of discrete characters was also critical for comparing crinoids, blastozoans, and other pentaradiate echinoderms until the work of Sumrall and Waters (2012) and Kammer et al. (2013). Disparity of this magnitude leaves little information for an objective basis for phylogeny.

FOSSIL ORGANIC MOLECULES

A substantial body of knowledge has been amassed to understand organic molecules preserved in sedimentary rocks. Preserved molecules have been an essential component of understanding the evolution of Proterozoic life (e.g., Summons and Walter, 1990; Brocks et al., 1999; Knoll, 2003; Gaines et al., 2009; Love et al., 2009) and have been essential for fingerprinting hydrocarbons (e.g., Tissat et al., 1978; Barakat et al., 2002). These studies have principally relied on isolated molecules from whole rock samples, which contrast with the present study that has isolated organic molecules directly from the fossil skeletons. In the former,

better-studied field, the structure of molecules is used, among other things, to infer the potential host organism or to characterize specific petroleum deposits. In contrast, the extractions of this study reveal the molecules still preserved within a fossil. Besides the work on fossil echinoderms, relatively few studies have extracted organic fossils directly from fossil. One example is Taylor et al. (2006), who extracted organic molecules from fossil plates. They identified oleanane from Cretaceous Bennettitales and Permian Gigantopteridales.

The biogeochemical pathways to preserve organic molecules in fossil echinoderms are, as yet, not fully understood. However, the fact that an original biological signal is preserved is demonstrated by the presence of fossil occurrences with different species of crinoids preserved in different colors (Bather, 1892; Laudon and Beane, 1937; Blumer, 1965; Lane, 1973; Wolkenstein, 2005; Wolkenstein et al., 2008; O'Malley, 2005, 2013).

LIVING ECHINODERM BIOMOLECULES

Echinoderms are the only animal phylum to produce anthraquinones and naphthoquinones (Stonik and Elyakov, 1988). At least 53 different naphthoquinones, anthraquinones, bianthrones, naphthopyrones, and complex related quinones have been identified in living echinoderms (e.g., Fox and Vevers, 1960; Fox, 1976; Gough and Sutherland, 1970; Kent et al., 1970; Francesconi, 1980; Rideout and Sutherland, 1981, 1985; Stonik and Elyakov, 1988; DeRiccardis et al., 1991; Takahasi et al., 2002). Some of these molecules are thought to function as defensive ingestion deterrents in modern echinoderms, i.e., it causes them to taste bad to potential predators.

In pioneering studies, Blumer (1951, 1960, 1962a, 1962b, 1965), Blumer and Omenn (1961), and Thomas and Blumer (1964) extracted polycyclic aromatic quinones (PAQ)

(fringelites) from Jurassic crinoids. Subsequently, various PAQs have been extracted from Triassic and Jurassic crinoids, and fringelites are now recognized as fossil hypericinoids (Wolkenstein et al., 2006, 2008; Wolkenstein, 2014). O'Malley et al. (2005, 2008, 2013) extracted organic molecules from Mississippian crinoids. Extracts were a mixture of organic molecules and were characterized using various techniques. Although the exact identity of individual molecules remains unknown in these mixtures, the extracts were compared with chemical standards that suggested that diagenetically altered quinone-like molecules were present. Regardless of the identity of each molecule in extract mixtures, O'Malley (2013) successfully used fluorescence excitation-emission spectroscopy (EEM) spectra to elucidate phylogenetic relationships among these fossil crinoids. Fluorescence spectroscopy is a non-destructive (with respect to the extracts), fast, and convenient approach to "fingerprint" the fluorophoric signature of extracts.

METHODS

Extraction of organic molecules was accomplished by placing finely ground solid sample into a glass fiber thimble within a Soxhlet apparatus, and methanol was continuously refluxed through the sample through dissolving echinoderm plates in hydrochloric acid and Soxhlet extraction. Extracts were concentrated into a pellet with a centrifuge. The solid pellet was further extracted in a 4:1 vol./vol. solution of acetone:methanol, decanted, and extracted again in methanol.

Fossil echinoderm extracts are water soluble and were subjected to various analytical measurements. In O'Malley et al. (2013) a combination of the Fluorescence Excitation Emission

Matrix Spectroscopy (EEM) and ESI-tandem mass spectroscopy were used. In contrast, here we demonstrate that robust data are derived using only EEM, which is relatively less expensive and less labor. Further, we demonstrate the potential of using EEMsfor addressing paleobiological problems.

EEM results are presented here. EEMs are an effective method to measure fluorescing compounds, such as PAQ. EEMs are collected by measuring emission scans with increasing excitation wavelengths, resulting in a contour plot that is highly detailed and can be used to distinguish and identify fluorescent compounds in complex mixtures (Christian et al., 1981; Lochmuller and Saavedra, 1998; Leiner and Wolfbeis, 1988; Coble, 1996; Stedmon et al., 2003; Cory and McKnight, 2005). The peaks in an EEM plot represent compound(s) with similar fluorescent properties and provide information on types of fluorophores present. EEM plots were generated using the MatLab code written by Rose Cory (Cory et al., 2005, 2010; Stedmon and Bro, 2008).

Fluorescence spectroscopy was conducted on a Varian Cary Eclipse. EEMs analyses were performed on samples such that absorbance at 254 nm was less than 0.05 (Lackowicz, 2006) and scanned with excitation wavelengths (λ_{ex}) = 240-450 nm (5 nm increments). Emission wavelengths (λ_{em}) were recorded in 2 nm increments between 300-600 nm. All EEMs were blank subtracted, corrected using correction files specific to the instrument, and normalized to Raman areas of DI water using a λ_{ex}=350 nm.

With Mississippian crinoids, O'Malley et al. (2008, 2013) demonstrated that the same organic molecules were consistently extracted from different positions (e.g., calyx and stem) along a single crinoid individual, and the extracts from fossil echinoderm specimens were different than extracts from the enclosing rocks. Because analyses of multiple specimens and

multiple parts of the anatomy of a specimen yielded the same results for Mississippian crinoids (O'Malley, et al., 2013), single specimens were evaluated in this study. Specimens are deposited in the Orton Geological Museum, The Ohio State University, as indicated in Table DR1.

Table DR1. Names, stratigraphy, locations, and specimen numbers for echinoderms from which organic molecules were extracted for the present analysis.

Class	Taxon	Geologic Period	Location
Asteroidea	*Stenaster* sp. OSU 54434	Bobcaygen Fm.; Ordovician	Kirkfield, Ontario
Blastoidea	*Pentremites* sp. OSU 54435	Indian Springs Shale, Mbr., Big Clifty Fm.; Mississippian	Sulpher, Indiana; NE¼ NE¼ SW¼, sec. 24. T3S, R1W
Crinoidea	*Elegantocrinus hemisphaericus* OSU 54436	Edwardsville Fm.; Mississippian	Boy Scout Camp, Monroe Co, Indiana; SW¼ SE¼ SW¼, sec., 8, T7N, R1E

Diploporita	*Holocystites* sp. OSU 54437	Massie Fm.; Silurian	Napoleon, Indiana; N39° 12’ 31.39”, W85° 18’ 53.75”
Echinoidea	cidaroid OSU 54438	unknown provenance	
Edrioasteroidea	*Isorophus cincinnatiensis* OSU 54439	Bellevue Fm.; Ordovician	Stonelick Creek, Cincinnati, Ohio; N39° 10’ 23”, W84° 07’ 35”

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