# Organic components of the skeleton of scleractinian corals – evidence from *in situ* acridine orange staining

PASCALE GAUTRET, JEAN-PIERRE CUIF, and JAROSŁAW STOLARSKI

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Scleractinian skeleton is composed of mineral and organic phases. Using staining techniques (acridine orange dye) Johnston's (1980) pioneering observations of intraskeletal organic envelopes in *Pocillopora damicornis* coralla can be extended to two other coral reef genera i.e., *Acropora* and *Favia*. The concept of biologically mediated growth of coral skeleton stands in opposition to the purely mineralogic concept of fiber growth of Bryan and Hill (1941) widely applied until recently in geological and paleontological literature. Presence of active mineralizing organic components within the skeleton explains various patterns of microstructural organization more accurately than the mineralogic concept of 'crystal growth competition' of Barnes (1970) alone. Biochemical degradation of intraskeletal organic matrices is considered to be involved in the initial diagenesis of coral skeleton, and may explain selective silicification of the late Cretaceous *Coelosmilia* sp. from Poland.

Key words: Biomineralization, diagenesis, skeletal matrices, acridine orange, staining techniques, Scleractinia.

*Pascale Gautret [gautret@geophy.geol.u-psud.fr] and Jean-Pierre Cuif [cuif@geophy.geol.u-psud.fr], Laboratoire de paléontologie, bât. 504, Université Paris XI-Orsay, F-91405 Orsay cedex, France.*
*Jarosław Stolarski [stolacy@twarda.pan.pl], Instytut Paleobiologii PAN, ul. Twarda 51/55, PL-00-818 Warszawa, Poland.*

## Introduction

A survey of present ideas concerning formation and growth of fibers of scleractinian skeleton reveals a paradoxical situation: two opposite concepts are simultaneously accepted, depending upon the research domain. Among geologists using coral skeletons as a sort of environmental archive, mineralization is considered as a 'physico-chemi-

cally dominated process' (Constantz 1986) following the highly influential paper of Bryan & Hill (1941). These authors suggested that the basic element of the coral skeleton, a fiber, is 'a single orthorhombic crystal of aragonite' formed without biological control (Bryan & Hill 1941: p. 84).

Among biologists, research dealing with the formation and growth of coral skeletons has been done for decades on the basis of an 'organic matrix' concept (Towe 1972). In this respect, the calcification model presented by Johnston (1980), although based on a single species – *Pocillopora damicornis* (Linnaeus, 1758), remains the only one that provided TEM and SEM ultrastructural images of organic materials associated with the mineral phase. In contrast to the physico-chemical model, illustrations provided by Johnston strongly suggest that fiber growth is biologically controlled in both transverse and longitudinal directions (e.g., Johnston 1980: figs. 7, 18). Unfortunately, in spite of the highy demonstrative character and great potential for explanation, Johnston's model did not exert much practical influence on earth-sciences literature, even in sectors that are primarily concerned with initial composition of biological structures (i.e., paleontology or isotopic investigations of paleoenvironments).

In this paper, we extend observation of the presence of fiber envelopes to two additional coral genera (*Acropora* and *Favia*) using a simple acridine orange dye staining technique. Because of the chemical affinity of this dye to organic components it is possible to discuss the biochemical nature of fiber envelopes, as well as possible relationships between fiber coating and the transverse lamination observed within fibers on etched sections. Significance of these data is discussed not only with respect to the question of fiber growth as a physico-chemical process or a biologically driven one, but also with respect to the possible role of the fiber organic matrix in early phases of diagenesis.

## Materials and methods

Our observations are based on recently collected specimens of *Acropora* sp. and *Favia fragum* (Esper, 1795) from Guadalupe Island (Caribbean Sea). Thin sections from the coral material were prepared and finely polished using water-dispersed aluminium oxide grinding powders. Diamond powders cannot be used in the polishing process since it is difficult to remove traces of the media in which diamond particles are dispersed (surface cleanness of specimen surfaces is a condition necessary for success in the staining process).

Acridine orange dye (Sigma-Aldrich prod. #A6014) was applied directly to the surface of thin sections. Epifluorescence of stained surfaces was observed under ultraviolet light using a Zeiss Standard microscope, Neofluor fluorine objectives, Zeiss mercury lamp, excitation filter (365 nanometer), and transmission cut-off filter (400 nanometer).

Stained parts of specimens were compared with microstructural data acquired from the same coral taxa using light etching of the polished surfaces. Etching was done using a solution of 0.1% formic acid and 3% glutaraldehyde (etching time 40 to 60 seconds). This kind of etching allows observation of differential solubility within the skeletal structures (observations performed using SEM Phillips 505).

In addition to Recent materials, we mapped polished surfaces of the specimen of the late Cretaceous *Coelosmilia* sp. from Poland using SEM Phillips 505 associated to EDS microprobe system LINK AN 10 000. This specimen was found in Piotrawin quarry (Central Poland) within deposits of marly siliceous chalk ('opoka') of late Campanian age (*Nostoceras hyatti* Zone = *N. pozaryskii* Zone of Błaszkiewicz 1980, see Burnett *et al.* 1990).

**Institutional abbreviations:** UPS, Université Paris XI-Orsay, Laboratoire de paléontologie, France; MNHN-BIM, Museum National d'Histoire Naturelle, Paris, Laboratoire Biologie des Invertébrés Marins; ZPAL, Institute of Paleobiology Polish Academy of Sciences, Warsaw, Poland.

## Results

Organic materials associated with Recent coral skeletons exhibit a positive response to acridine orange (Fig. 1A, B, D–H). Stainable organic compounds can be allocated to three sites within microstructural organization of corals: centers of calcification, fiber envelopes, and growing fronts of fibers. SEM/light microscope observations of etched surfaces or ultra-thin sections, respectively, appeared very useful to interpret color patterns of the dye and establish correspondance between these stained portions and the microstructural organization of the skeleton of coral species investigated.

**Skeleton of the Recent *Acropora* sp.** — The main part of the *Acropora* skeleton is built of minute scale-like units, ca. 5×15μm in transverse section. These units are easily visible on the surface of various skeletal elements (Fig. 2A). Individual scales are composed of bundles of fibers (Fig. 2C). Cross sections demonstrate that the same scaly microstructural organization is common for the entire skeleton, not only limited to the external surface. A polarized light-microscope picture (Fig. 1C) shows a clear correspondance between scaly units forming lamellae organized around microstructurally separate centers of calcification, and fine morphology of the external surface. When etched with formic acid, cross sections show that the peripheral zone of scale-like units is less susceptible to etching in comparison to the fibrous core. This was previously interpreted as indicating the presence of organic matrices at the outer side of the units (Cuif *et al.* 1977).

Sections stained with acridine orange (Fig. 1A, B) reveal the location of calcification centers as elongated or rounded patches strongly contrasting with a very bright green or yellow-green fluorescence. Also the typical scaly pattern of the acroporid skeleton is emphasized by the action of the fluorescent dye. The bright fluorescence of borders surrounding scale-like units indicates the presence here of organic coating which delimits invidual units. The fluorescent color of this coating is mostly yellow-green to yellow in areas close to calcification centres, but becomes orange in more remote areas (Fig. 1B).

In addition to envelopes surrounding fibrous units and calcification centers, the most external, newly formed zone of the skeleton also shows a strong positive response to acridine orange, with very intense orange fluorescence (Fig. 1A). This attests to the high concentration of organic compounds within these still newly, but completely calcified zones.

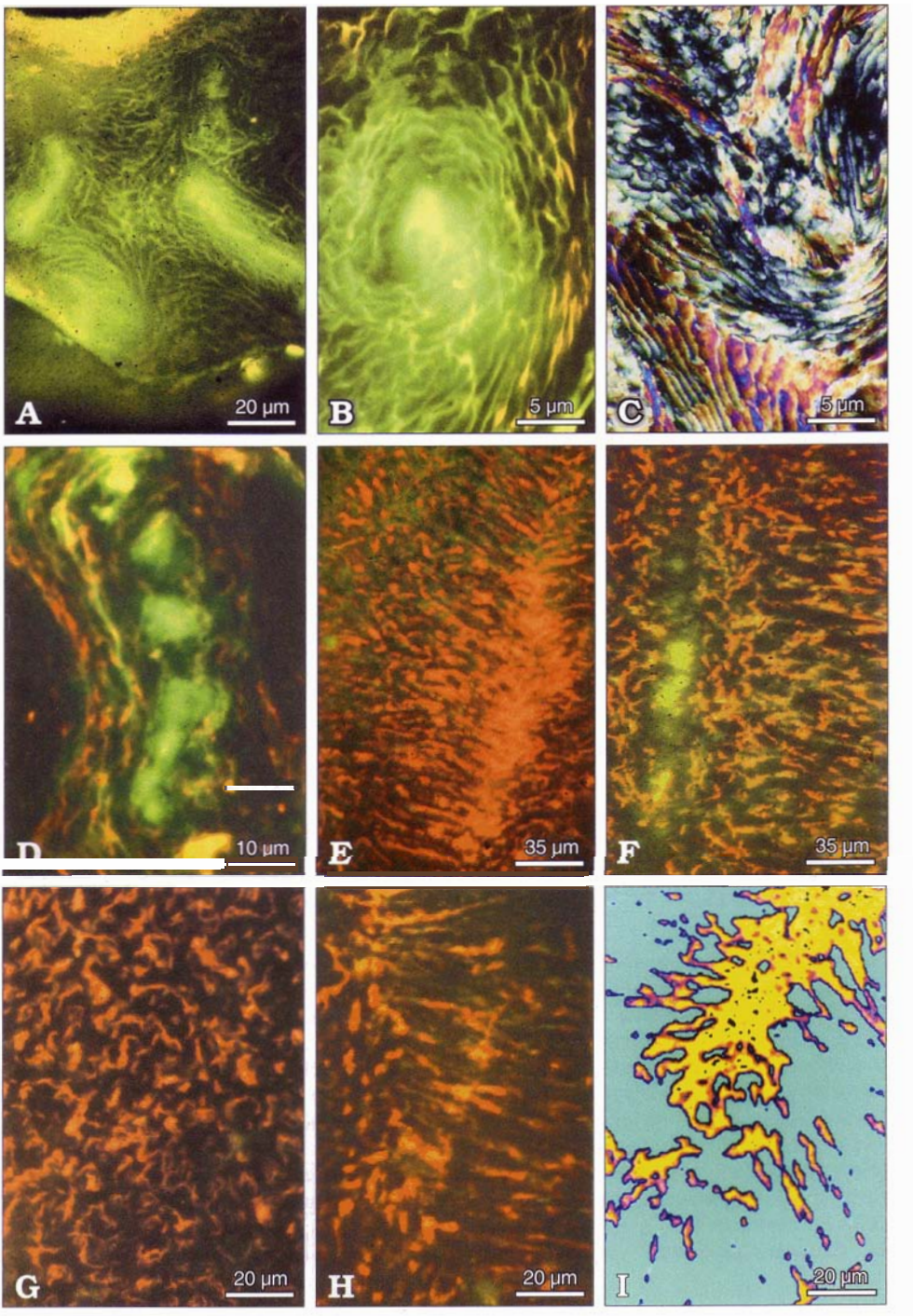
**Skeleton of the Recent *Favia fragum*.** — Skeletal microstructure in *Favia fragum* was recently described in detail by Cuif & Perrin (1999) and here only few SEM pictures of the skeletal microstructure are reported to provide a source for comparison with fluorescent patterns in acridine-stained thin sections (Fig. 2D–G).

Both calcification centers and fibers of *F. fragum* positively respond to acridine orange, but with a very strong contrast in fluorescent colors. Calcification centers exhibit bright green fluorescence (Fig. 1D, F), forming a dotted line in the medial axis of transversely cut septa. Fiber envelopes oriented normal to the external border of septa are mostly intense red (Fig. 1E, F–H). Distances between successive semi-parallel red lines (if fibrous structure is cut longitudinally; Fig. 1E, F, H), or distances between the spaces delimited by the vermiculate network in transverse cuts (Fig. 1G) suggest that acridine-stained envelopes coat bundles of fibers rather than individual fibers.

The outer parts of fibers at the external border of septa also demonstrate a zone of very intense, red response to acridine orange (Fig. 1E). In the free part of septa inside the calice this intensively stained zone is visible as apparently concentric red lines (Fig. 1G), which are comparable with the microarchitecture of septa i.e., bundles of fibers which are oblique to this surface, and not coinciding with successive growth lines (Fig. 1F). The red zone marking the distal end of fibers does not stop at the border with the calicular wall but is continuous between adjacent septa.

**Skeleton of the Cretaceous *Coelosmilia*.** — A skeleton of late Cretaceous *Coelosmilia* sp. from Poland, is neomorphically altered into calcite. Neomorphic calcite crystals with partly fibrous appearance (remainders of original fibrous structure) have boundaries that clearly coincide with boundaries of original bundles of skeletal aragonite. Remarkably, in addition to this neomorphic calcitization, the skeleton is partly silicified (Figs. 2H–J, 3A, B). Microprobe mapping (Fig. 1I) emphasizes the location of silica with respect to the septal microstructure. Zones of silicification were found primarily in the mid-septal zones (i.e., where calcification centers were originally located), as very fine sheaths surrounding neomorphic calcitic structures,

Fig. 1. **A, B.** Acridine orange staining of organic components within the skeleton of *Acropora* sp. (UPS/NP2240); Recent, Guadeloupe Island (Caribbean Sea, several meters). **A.** Organic components in growing portions of the skeleton show a bright yellow chromatic response, internal zones of centers of calcification are pale yellow, whereas organic envelopes of scaly units incorporated within deeper parts of the skeleton are green to yellow. **B.** Enlarged region around calcification center. **C.** Ultra-thin section (polarized light) of the skeleton of *Acropora* sp. (UPS/NP2240); Recent, Guadeloupe Island (Caribbean Sea, several meters). Polarized light emphasizes the crystal-like behaviour of the units defined and enclosed by colored organic envelopes on B. **D–H.** Calcification centers and organic envelopes in *Favia fragum* (Esper, 1795); UPS/NP2117; Recent, Guadeloupe Island (Caribbean Sea, several meters). **D.** Calcification centers on the septal growing edge (transverse section) are arranged in short linear series (compare Fig. 2G). They exhibit bright green-yellow fluorescence, whereas organic components on lateral growth surfaces show mostly red fluorescence. **E, F.** Fluorescence of organic components of calcification centers changes from intense orange to red at the septal growth front (**E**) to yellowish within deeper parts of septa (**F**). Skeletal fibers adjacent to calcification centers form distinct linear units. **G, H.** Stained organic envelopes of fibers in transverse (**G**) and longitudinal section (**H**); compare also Fig. 2G. **I.** Microprobe mapping of silica (yellow) in the calcitic skeleton of *Coelosmilia* sp.; ZPAL XIX/1/B32; Late Cretaceous (late Campanian, *Nostoceras hyatti* Zone), Piotrawin (Central Poland). Distribution of the silica matches the pattern of occurrence of organic coatings of fibers in *Favia fragum*.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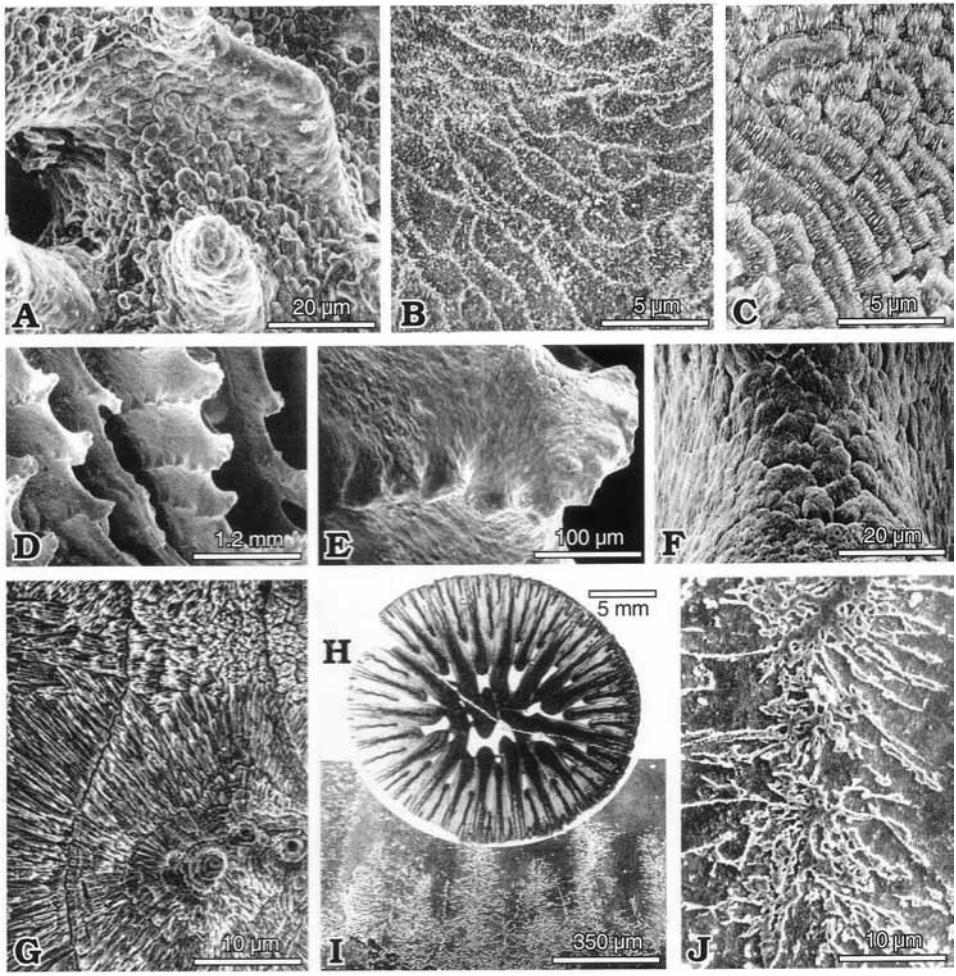


Fig. 2. **A–C.** *Acropora sp.* skeletal surface morphology and etched sections (UPS/NP2236); Recent, Guadeloupe Island (Caribbean Sea, several meters). **A.** Scaly-like units cover entire surface of the skeleton. **B, C.** Polished and etched surfaces. Zones of resistance to acid etching in external parts of skeletal units correlates with position of organic coatings detected by acridine orange staining (compare Fig. 1B). **D–G.** *Favia fragum* (Esper, 1795); Recent, Guadeloupe Island (Caribbean Sea, several meters). **D, E.** Calcification centers are located in short series, at the top of denticulate septal structures; MNHN-BIM/631, Milne Edwards coll. **F.** Fiber growth edges at the surface of septum; MNHN-BIM/631, Milne Edwards coll. **G.** Polished and etched section of septum. Due to divergence from opposite calcification centers, groups of fibers are sectionned transversally (top view) or longitudinally (low part of the picture); UPS/NP2758. **H–J.** Morphology of the transverse sectioned corallum of *Coelosmilia* sp. Zones of silicification can be correlated to organic coatings of fibers in Recent corals; ZPAL XIX/1/B32; Late Cretaceous (late Campanian, *Nostoceras hyatti* Zone), Piotrawin (Central Poland).

and, occasionally, in contact zones between septa. Orientation of these sheaths, normal to the septal margin, precisely corresponds to the growth direction of fibrous units (Figs. 2J, 3A, B).

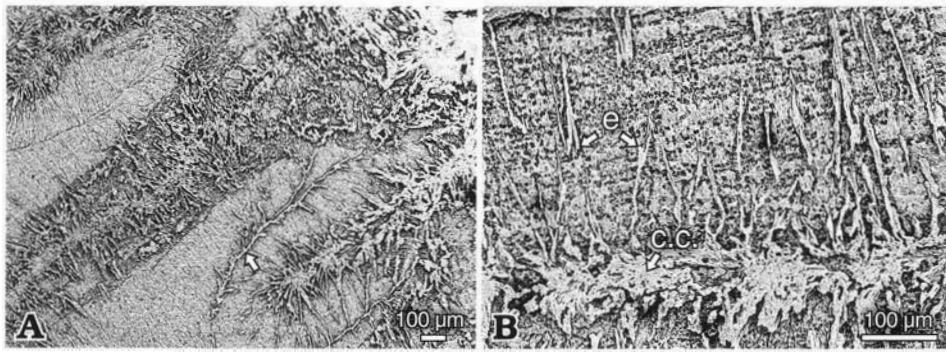


Fig. 3. Etched transverse section of corallum of *Coelosmilia* sp. (ZPAL XIX/1/B32) with excellently preserved silica structures (positve relief on both micrographs). **A**, **B**. Zones of silicification are limited primarily to the mid-septal zones (arrowed as c.c. on **B**). Fine sheaths of silica surround also neomorphic calcitic structures ('e' arrows on **B** indicate border of an 'envelope') and, occasionally, are present in contact zones between septa (arrow on **A**). Late Cretaceous (late Campanian, *Nostoceras hyatti* Zone), Piotrawin (Poland).

## Discussion

Fibers were described in coral skeleton as early as 1882 (Pratz). At the same time attempts were made to understand coral skeletogenesis and to use microstructural characters of the skeleton for taxonomy. Heider's (1881) cellular theory of skeletogenesis was rejected by Koch (1882) who proposed an extracellular model (Koch's taxonomic suggestions concerning significance of organization of coral skeletal fibers were, unfortunatelly, unsatisfactory). Interestingly, Ogilvie (1896), who undoubtly was the most aware of the diversity of coral microstructures at this time, supported the cellular theory as the only plausible mechanism to explain the biologic control of skeletogenesis.

The first biochemical evidence for an extracellular control by a specifically secreted organic matrix in corals was proposed by Goreau (1959), who described a mucopolysaccharidic layer at the outer side of the basal ectodermal tissue of the coral. This organic layer was long admitted to act as a simple 'template' for aragonite crystallization (Milliman 1974).

Johnston (1980), in a study that still remains the most innovative analysis of calcification patterns in a coral species (i.e., *Pocillopora damicornis*) emphasized the importance of a 3 mm thick primary layer that creates a continuous reticulum ('meshwork') at the growing front of the skeleton. He also illustrated envelopes that surrounded fibers in *P. damicornis*, but probably due to his preparative technique, he considered that the organic meshwork 'simply disappear' from coral skeletons after having played the leading role in the crystallization at the growing front of fibers.

### Distribution of organic matrices in coral skeletons and variability of chromatic responses

With respect to the Johnston's model, two main points should be emphasized:

(1) Presence of envelopes surrounding microstructural components, the main result of the Johnston (1980) research, is confirmed by acridine orange stainings in *Acropora*

sp. and *Favia fragum*. Staining of polished surfaces allows the whole length of fibers to be submitted to reactive dye, which differs from Johnston's (1980) fixation technique that was only affected at the growth surface, resulting in an apparent disappearance of organic matrices inside the skeleton. Acridine staining techniques unambiguously show that organic matrices are also present within the deeper parts of the skeleton. Microstructural patterns after enzymatic and acidic etching of the skeleton (Cuif *et al.* 1997), as well as analysis of soluble/dissoluble organic components of the scleractinian skeleton (Gautret & Marin 1992; Gautret *et al.* 1997), provide consistent evidence that mineralizing matrices remain entrapped within crystal-like fibers and are not removed immediately below the growing front.

(2) Fluorescent patterns shown by acridine orange stained thin sections confirm another important conclusion of Johnston (1980) concerning biochemical distinctness of organic matrices present at the fiber growth front ('organic meshwork'). Generally, *Acropora* and *Favia* plainly exhibit an intense coloration at the growing parts of the skeletons (Fig. 1A, E). Repetitive secretion of micron-scaled layers of skeletogenetic matrices becomes a plausible mechanism for coral skeletogenesis, an hypothesis that is supported by incremental growth of coral fibers (Sorauf & Jell 1977 in *Desmophyllum*), or simply pictured (Ogilvie 1896 in *Mussa*).

## Fibers and calcification centers: histochemical differences evidenced by acridine orange

Organic envelopes of fibers in *Acropora* and *Favia* stained with acridine orange show different fluorescence responses (yellow-orange in *Acropora*, and red in *Favia*), suggesting different matrix compositions in the two genera. Differences in biochemical composition of mineralizing matrices have earlier been suggested to be useful in taxonomy (Cuif *et al.* 1997). The chromatic response of organic matrices present in various regions of fibrous part of skeleton is similar, though more intense on growth surfaces and marginal parts of envelopes that coat bundles of fibers (intense yellow-orange in *Acropora*, and intense red in *Favia*). Comparable color patterns of organic matrices within fibrous parts of the skeleton suggest their similar biochemical composition.

Staining techniques confirm that matrices associated with calcification centers and fibers differ in their biochemical composition, as previously suggested on a physical basis (Cuif & Dauphin 1998). We observed significantly different chromatic responses between centers and surrounding fibers within the skeleton of two genera (fluorescence of calcification centers is bright green or yellow-green in *Acropora*, and bright green in *Favia*).

## Biochemical significance of *in situ* acridine orange staining

Acridine orange is a cationic dye, the chromatic response of which is affected not only by the gross secondary structure of the substrate, but also by concentrations of adsorbed molecules, pH, temperature and ionic strength. It is commonly admitted that the fluorescence wavelength emited by acridine stainings depends on the distance between bound molecules: when acridine molecules are very closely fixed so that the dimeric form is possible, red fluorescence is obtained, whereas when the density of the fixed molecule is lower, the monomeric form produces green fluorescence. More detailed observations

show that differences in chromatic response between the fiber growth surface and the internal parts of fibers may be due to changes in density of sites for acridine fixation.

At the growing surface, the acidic glyco-proteic mineralizing meshwork is not completely infilled with aragonite microcrystals, and a great number of negative sites are available for fixation of positively charged molecules. However, in underlying growth increments most of the possible sites are already occupied. This may explain why in deeper parts of the skeleton there is no visible difference between successive growth layers.

### Organic envelopes and the diagenetic process: preliminary notes

The very fine pattern of silica deposition in the Cretaceous *Coelosmilia* sp. as confirmed by microprobe mapping, perfectly reflects the position of organic envelopes in extant corals such as *Favia*. We suggest that the decay of organic matrices which started right after the completion of formation of the mineral phase, caused a lowering of intraskeletal pH values, thus creating a preferential diagenetic microenvironment for the deposition of silica. After decay was completed, pH returned to higher values and the deposition of silica stopped. Similarly, preferential silicification of shells in limestones was attributed to the presence of organic matter in shell matrices (Wetzel 1957). Mechanism involving degradation of organic matter in carbonate sediments, increasing of $CO_2$ activity, and hence, drop in pH was invoked to explain silicification and carbonate dissolution (Siever 1962; see also Knoll 1985). As noted above, the specimen of *Coelosmilia* sp. investigated is neomorphically calcitized and several morphological aspects of the original microstructural organization are retained. To what extent this early silica deposition could be related to the particular preservation of skeletal microstructures in *Coelosmilia* sp. requires more extensive studies of additional material. Noteworthy, similar microstructural pattern has recently been reported in Cretaceous aragonitic corals from Gosau, as well as remnants of organic matter within the skeleton (Sorauf 1999: fig. 4: 4). Sheaths of organic matrix preserved within the aragonitic skeleton of *Rennensismilia complanata* (Goldfuss, 1826) are almost identically arranged around bundles of aragonitic fibers as silica sheats enclosing neomorphic calcitic structures in *Coelosmilia* sp. Clearly, in addition to their role as growth controlling agents during formation of the corallum, organic compounds appear to be important factors in early diagenesis, probably with long term consequences.

## Conclusions

- The presence of the organic envelopes surrounding skeletal fibers that form coral skeleton is confirmed in new scleractinian taxa (i.e., *Acropora*, *Favia*). Although various factors may affect chromatic reaction of acridine orange dye, differential staining between fibers and envelopes suggests a biochemical difference between these two organic phases controlling fiber growth. These observations contribute to the compartmental theory of biomineralization (Bevelander & Nakahara 1980).
- Positive response of the entire skeleton to acridine orange staining indicates that mineralizing matrices persist entrapped within crystal-like fibers. This supports pre-

vious interpretations of the presence of organic-rich and organic-depleted zones within the coral skeleton based on differential solubility of the mineral phase.
- Acridine staining confirms a biochemical difference between calcification centers and fiber organic envelopes.
- The concept of 'organo-mineral composition of coral fibers' provides a new perspective for discussion of diagenesis of scleractinian skeletons. It is suggested that decomposing mineralizing matrices play a significant role in the early diagenesis and microenvironmental conditions resulting from decay of fiber envelopes.

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## Substrancje organiczne w szkielecie Scleractinia oraz ich wykrywanie metodą barwienia oranżem akrydynowym

PASCALE GAUTRET, JEAN-PIERRE CUIF i JAROSŁAW STOLARSKI

### Streszczenie

W dotychczasowej literaturze geologiczno-paleontologicznej poświęconej Scleractinia interpretacje dotyczace budowy mikrostrukturalnej szkieletu oparte były o model zaproponowany przez Bryan i Hill (1941). Według tego modelu, tworzenie włókien szkieletu korali tłumaczą reguły fizyko-chemiczne obowiązujące w trakcie czysto abiotycznej krystalizacji – np. „wzrost kompetencyjny" kryształów (Constantz 1986), zaś biologiczna regulacja sprowadza się jedynie do inicjowania centrów kalcyfikacji („zarodków krystalizacji"). Tymczasem, w literaturze biologicznej, wzrost szkieletu różnych organizmów, w tym korali, rozumiany był od dawna w kategoriach uzupełniających się aktywności fazy mineralnej i organicznej szkieletu. Szczególnie ważne były pod tym względem badania Johnstona (1980), który wykazał, że u *Pocillopora damicornis* obecne są organiczne otoczki pęków włókien, które wnikają wgłąb szkieletu oraz, że w marginalnej, wzrostowej jego części włókna szkieletowe przenika „organiczna siateczka" (*organic meshwork*).

W niniejszej pracy potwierdzamy obserwacje Johnstona (1980) dotyczące występowania organicznych otoczek pęków włókien szkieletowych. Ich obecność stwierdzamy w szkieletach współczesnych korali rafowych *Acropora* i *Favia* za pomocą metody barwienia oranżem akrydynowym. Na podstawie odmiennych widm fluorescencyjnych zabarwionych struktur organicznych wyróżniamy trzy główne rejony w szkielecie badanych korali: (1) centra kalcyfikacji, (2) włókna szkieletowe tworzące podstawową część

septum, oraz (3) marginalną, wzrostową strefę szkieletu. Różnice w reakcji chromatycznej między zawartymi w tych rejonach strukturami organicznymi, szczególnie między organicznymi składnikami centrów kalcyfikacji i otoczkami pęków włókien, sugerują ich odmienny skład biochemiczny. Odmienne barwy fluorescencyjne otoczek pęków włókien u *Favia* i *Acropora* sugerują, że różnice biochemiczne między tymi strukturami mają podłoże taksonomiczne.

Selektywne trawienie szkieletu z użyciem enzymów i kwasów organicznych, potwierdza zaś drugą obserwację Johnstona dotyczącą występowania substancji organicznej wewnątrz włókien szkieletu. Trawione w ten sposób włókna ukazują obecność mikronowej grubości warstewek organicznych, rytmicznie odkładanych w ich wnętrzu.

Występowanie wewnątrzszkieletowych substancji organicznych ma znaczenie przy reinterpretacji szeregu tradycyjnych poglądów na temat diagenezy szkieletu korali. Rozpatrując czynniki wpływające na diagenezę należy uwzględniać nie tylko takie czynniki geochemiczne jak stabilność aragonitu/kalcytu w środowisku wodnym, obecność, skład i temperaturę roztworów wewnątrzporowych, ale również czynniki biochemiczne takie jak występowanie, przestrzenna konfiguracja i skład chemiczny substancji organicznych przenikających strukturę szkieletu. Substancje te, podlegają już w trakcie wzrostu szkieletu oraz po śmierci korala przemianom biochemicznym i przyczyniają się do kształtowania specyficznego mikrośrodowiska diagenetycznego w przestrzeniach wewnątrzszkieletowych. Przestrzenne ułożenie krzemionkowych otoczek pęków włókien oraz obecność krzemionki w strefie środkowej septum (rejonie występowania centrów kalcyfikacji) w skalcytyzowanym szkielecie *Coelosmilia* sp. z późnokredowych opok okolic Kazimierza Dolnego odzwierciedla rozmieszczenie struktur ogranicznych u współczesnych korali takich jak np. *Favia*. Sugeruje to, że wytrącenie krzemionki z nasyconego nią roztworu było następstwem rozkładu wewnątrzszkieletowych struktur organicznych i lokalnego obniżenia pH.