

## Green-fluorescent proteins in Caribbean corals

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### Abstract

Fluorescent pigments in several Indo-Pacific and Caribbean anthozoans have recently been identified as proteins related to the green-fluorescent protein (GFP) of the hydromedusa *Aequorea victoria*. Here we show that GFP is widely distributed in many Caribbean species. The fluorescence excitation and emission spectra for the pigment are similar to those reported elsewhere for coral and noncoral GFP and the fluorescence quantum yield is estimated to be 35%. Spectral and molecular characterization of the isolated protein clearly show it to be GFP, and laboratory and in situ fluorescence measurements and Western blot analysis show that it is widespread. Bathymetric studies of GFP content using Western blots for the ecologically important congeneric corals *Montastraea faveolata* and *Montastraea cavernosa* show that there is no significant correlation between depth and GFP concentration. Nucleotide sequence data of GFP from *M. faveolata* and *M. cavernosa* show 88.2% sequence homology with each other and 46.4% homology with *A. victoria* GFP, whereas the percent homology with *A. victoria* at the amino acid level was 31.1 and 28.4% for *M. cavernosa* and *M. faveolata*, respectively, and 82.7% with each other. Measurements of reflectance and of the excitation spectrum for chlorophyll fluorescence in GFP-containing corals indicate that GFP absorption, emission, and reflection have negligible impact on the level of solar radiation reaching the zooxanthellae and therefore play no role in coral photosynthesis by either addition or removal of photons.

Zooxanthellate corals contain a number of fluorescent substances, one of which is chlorophyll in the endosymbiotic dinoflagellate of the genus *Symbiodinium* (=zooxanthellae). This photosynthetic pigment contributes a red fluorescence, with peaks near 685 and 735 nm, from the gastrodermal cells in which the zooxanthellae reside. Other pigments fluoresce at shorter wavelengths and are usually contained in the epithelial cells of the cnidarian host (Kawaguti 1944; Logan et al. 1990; Mazel 1995; Salih et al. 2000), although they may at times be located in gastrodermal tissue (Schlichter et al.

1985; Salih et al. 1998). In vivo measurements of the fluorescence excitation/emission spectra of a variety of anthozoan species in the Caribbean indicate that a limited number of spectrally distinct pigments are responsible for the wide range of subjective fluorescence color responses that can be observed (Mazel 1995, 1997b). These pigments may occur singly or in combination, in a variety of macroscopic spatial patterns in the coral tissue. The function of the fluorescence has variously been ascribed to (1) providing photoprotection in high-light conditions (Kawaguti 1969; Salih et al. 2000); (2) enhancing photosynthesis in low-light conditions (Schlichter and Fricke 1990); or (3) both, depending on the positioning of the fluorescent pigment relative to the zooxanthellae (Salih et al. 1998; Dove et al. 2001). The fluorescence can contribute to the appearance of corals under daylight illumination (Limbaugh and North 1956; Marden 1956; Mazel and Fuchs 2003) or may only be evident under excitation with light of appropriate wavelengths (Catala 1959; Logan et al. 1990).

Several fluorescent proteins in corals, based on fluorescence emission spectra and molecular data, have been iden-

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tified (Mazel 1997b; Matz et al. 1999; Dove et al. 2001; Labas et al. 2002), but here we are concentrating on GFP with an emission maximum in the range of 500 to 518 nm. Using biophysical, biochemical, and molecular techniques, we have identified one of the host pigments in several Caribbean corals as a green-fluorescent protein (GFP). A variety of spectral measurements argue against either a photoprotective or photoassistive function for the GFP. Our results complement and extend recent work (Labas et al. 2002) on green fluorescent proteins of Caribbean scleractinian corals by using molecular and biophysical approaches to confirm the presence of these proteins and to investigate their ecological role in the photobiology of corals.

## Methods

*Field sites and sample collection*—Coral specimens were collected from depths ranging from 3 to 30 m at reef sites (Rainbow Garden, 3 m; Horseshoe Reef, 8 to 10 m; North and South Perry Reef, 18 m; and Bock Cay, 23 to 30 m) around Lee Stocking Island (LSI), Bahamas (23°46.5'N, 76°05.5'W). Samples were maintained in aquaria with running seawater prior to analyses. The aquaria were shaded by using Vexar plastic netting as a neutral density screen to match the maximum level of natural sunlight to the irradiances measured at the collection depth of the corals.

*Fluorescence microscopy*—Samples of the coral *Montastraea cavernosa* were collected from 3, 10, 18, 23, and 30 m; fixed in 3% glutaraldehyde and filtered (0.22  $\mu$ m) seawater; and transported to the University of New Hampshire where dehydration and decalcification (5% ethylenediaminetetraacetic acid [EDTA]) were carried out. Samples were then embedded in optimum cutting temperature (OCT) compound (Miles Scientific) at  $-50^{\circ}\text{C}$ , and 5- $\mu$ m frozen sections were mounted on microscope slides and observed in a Zeiss Axioptot microscope at 100 $\times$  with an excitation filter of 460 to 500 nm bandpass and a barrier filter of 510 to 560 nm bandpass.

*Spectroscopy*—Fluorescence emission spectra were measured in situ with a diver-operated spectrofluorometer (Mazel 1997a). Using two different versions of the instrument, the coral surface could be illuminated either with light from a broadband source fitted with user-selectable interference filters (400, 440, or 488 nm center wavelength) of 10 nm bandpass (full width at half maximum, FWHM), or with high intensity blue light-emitting diodes (LEDs) with peak emission at circa 470 nm (26 nm FWHM). The instrument's measurement probe excluded ambient light. The light emitted by fluorescence, along with reflected excitation light, was carried to the single-board spectrometer (Ocean Optics Model S2000) in the underwater housing. Spectral fluorescence in the range of 500 to 750 nm could be measured with this instrument. The measurement spot size was approximately 0.25 cm<sup>2</sup>. Data were stored in nonvolatile memory and were downloaded to a laboratory computer after each dive. An alphanumeric keypad on the instrument enables the operator to enter brief notes, such as specimen identification and description of specimen condition or location. This informa-

tion, along with a depth reading and several instrumental readings that provide a quality check on performance, was stored along with the measurement data.

This instrument was also used to measure the reflectance of corals. For reflectance measurements, a combination of blue, white, and red LEDs in the measurement probe head provided broadband illumination from 390 to 800 nm. The light from the LEDs passed through a holographic diffuser (Physical Optics) and provided even illumination over the measurement area. A reading of the incident light provided by the measurement probe was made by placing a Spectralon (Labsphere) reference surface in the measurement plane. Readings of the light reflected from coral specimens were divided by the incident light reading to compute reflectance. New reference measurements were made at least every 15 min to guard against slow instrument drifts. In the case of corals containing fluorescent pigments, the light measured by the instrument was comprised of both reflected and fluoresced photons.

Fluorescence excitation and emission spectra were measured in vivo in the laboratory from freshly sampled specimens with a SPEX FluoroMax-2 spectrofluorometer (Jobin Yvon) fitted with a fiber optic probe. The measurement spot size was approximately 1 cm<sup>2</sup>. Absorption and fluorescence excitation/emission spectra of extracts and purified protein were measured with an Aminco DW2c spectrophotometer and an Aminco AB2 spectrofluorometer, respectively.

We also used fluorescence spectroscopy in the laboratory to investigate the possible role of GFP in coral photosynthesis. Measurements of the excitation spectrum for chlorophyll fluorescence (emission wavelength 690 nm) were made with the SPEX FluoroMax-2 to determine if GFP serves a photoprotective function in corals, or if it enhances photosynthesis. If a pigment were serving a photoprotective role, it would be removing photons that would otherwise reach chlorophyll and its antenna pigments. In that case the absorption (excitation) spectrum for the pigment should appear as a minimum in the chlorophyll excitation spectrum. Conversely, if any pigment, fluorescent or otherwise, were contributing energy to photosynthesis, we would expect to see a peak in the excitation spectrum for chlorophyll fluorescence corresponding to the absorption (excitation) peak for that pigment.

*Fluorescence lifetime measurements*—We examined fluorescence decay kinetics using laser-based single photon counting time-correlated fluorometry (O'Connor and Phillips 1984) and phase shift fluorometry. The first technique permits precise analysis of multicomponent kinetics in the laboratory, while the second allows rapid measurements of average fluorescence lifetimes in the field. In the first technique, frequency-doubled pulses (duration 2 ps, wavelength 410 nm) from a Ti-Sapphire mode-locked laser were used to excite fluorescence. The fluorescence emission was selected by a green interference filter (central wavelength 510 nm, bandwidth 10 nm FWHM), recorded by an ultrafast multichannel plate photomultiplier, and processed with a discriminator and a time-to-amplitude converter interfaced to a multichannel analyzer. Fluorescence kinetic profiles were acquired in the time window of 20 ns with the maximum signal

of 32,000 counts. Two exponential decay components were sufficient to fit the measured fluorescence profiles. Under field conditions, fluorescence lifetimes were measured with a phase shift fluorometer (Ciencia). This instrument excites the sample with a light source sinusoidally modulated at high frequency. The phase lag of the fluorescence signal relative to the excitation source provides a measure of the average lifetime of the fluorescent components in the subject.

*Isolation, purification, and characterization of protein and antibody production*—Surface tissue was stripped from freshly collected specimens of the coral *Leptoseris cucullata* by using a Teledyne water pik and 10 mM Tris pH 8 buffer. Proteins were precipitated from the resulting tissue blastate by adding 100% ice-cold acetone to aliquots, resulting in a final concentration of at least 70% acetone. Acetone precipitates were held in a  $-20^{\circ}\text{C}$  freezer for at least 1 h and then centrifuged at  $3,000 \times g$  for 5 min. The resulting supernatant was discarded, and the pellet was washed briefly with 100% acetone to remove water. Following a second centrifugation at  $3,000 \times g$ , the supernatant was discarded, and the pellet was allowed to dry. Samples were kept frozen and refrigerated for transport to Rutgers University.

For protein purification, acetone precipitates were initially solubilized for at least 1 h in 5 to 10 ml 10 mM Tris pH 8 buffer containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) at 100  $\mu\text{M}$  final concentration. Nonsolubilized material was removed by centrifugation at  $1,000 \times g$ . Ammonium sulfate (1.5 M final concentration) was added to the resulting supernatant, which was then centrifuged at  $10,000 \times g$  for 30 min. The precipitated protein pellet was discarded, and the supernatant was kept for subsequent separation by chromatography.

The ammonium sulfate supernatant was applied to a phenyl-sepharose 6 fast flow (Sigma) hydrophobic interaction column and washed with 2 to 3 column volumes of 1.5 M ammonium sulfate in 10 mM Tris pH 8. Subsequently, the GFP protein was eluted with 10 mM Tris pH 8 alone, resulting in 2 to 4 GFP-containing fractions of 2 ml each. These fractions were pooled and frozen at  $-80^{\circ}\text{C}$ . Pooled fractions were applied to a model 491 prep cell (Bio-Rad) continuous elution electrophoresis system in which the non-denaturing acrylamide gel system was discontinuous (4% stacking/6% resolving) and based on an Ornstein–Davis buffer system (pH approaching 9.5). GFP fractions eluted from this system were concentrated in Centricon-10 centrifugal filter units (AMICON) and aliquots were taken for protein analysis (BCA, Pierce Chemical).

Size exclusion chromatography was performed using high performance liquid chromatography (HPLC, Shimadzu) with a  $300 \times 7.8$  mm Bio-Sil SEC-125 column (Bio-Rad). The elution buffer was 50 mM  $\text{PO}_4$  pH 6.8/100 mM NaCl/0.01 Na-Azide. Protein purity was further assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of denatured protein aliquots followed by silver staining. The protein was judged to be pure when only a single protein band was observed. Antiserum to the GFP protein extracted from *L. cucullata* was prepared by injecting rabbits with purified protein and exsanguination after 8 to 10 weeks.

Circular dichroism (CD) spectroscopy was performed on pure protein samples of GFP with an AVIV model 60DS (AVIV Instruments). Deconvolution of CD spectra was done using CDNN software (version 2.1, Bohm).

*Western blot*—Soft tissue from individual corals collected from 3, 10, 18, 23, and 30 m ( $N = 3$  at each depth) was removed by scraping with a nylon brush and homogenized at  $4^{\circ}\text{C}$  in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.5) with dithiothreitol (DTT) and PMSF to prevent protein oxidation and protease activity respectively. The homogenate was then centrifuged at  $500 \times g$  for 20 min, and the supernatant was saved for analysis of protein (Bradford 1976).

From the preparation described above, protein extracts of samples of equivalent biomass were separated on SDS gradient (4 to 15%) polyacrylamide gels with a modified Laemmli buffer system and transferred to nitrocellulose as described by Lesser et al. (1996). The transferred proteins were immunoblotted using the polyclonal antibodies against GFP described above and visualized using secondary antibodies with a peroxidase label. The immunoblots were scanned and the optical density of the positive bands measured by means of the gel scanning procedures described in NIH Image (version 1.61) and based on a calibrated gray scale. Optical densities were log transformed before statistical analysis.

*DNA sequencing*—Samples of *Montastraea cavernosa* and *Montastraea faveolata* were collected at 10-m depth from LSI and stored frozen in RNALater (Ambion) for transport to the University of New Hampshire. Soft tissue from the corals was removed from an area  $\sim 2.5$   $\text{cm}^2$  by scraping with a nylon brush. The removed tissue was collected in microcentrifuge tubes, and ribonucleic acid (RNA) was extracted from the samples by using a modified TRIzol technique (Life Technologies) that included a lithium chloride incubation. Standard TRIzol protocol for RNA extraction was performed through the ethanol wash stage. After centrifugation of the sample and ethanol, the supernatant was aspirated off and the pellet dissolved in 100  $\mu\text{l}$  of milliQ  $\text{H}_2\text{O}$  and mixed by vortexing. An equal volume of 12 M LiCl was then added, vortexed, and incubated for 30 min at  $-20^{\circ}\text{C}$ . After this incubation, the samples were centrifuged at  $12,000 \times g$  for 15 min at room temperature and washed with 0.5 ml 75% ethanol. The ethanol was aspirated, and tubes were left open to allow the pellet to air dry. The RNA was then reconstituted in 50  $\mu\text{l}$  of milliQ  $\text{H}_2\text{O}$  and visualized on an agarose gel. Concentration and purity were determined spectrophotometrically using the A260/A280 ratio.

Single-strand cDNA was synthesized by using the SuperScript first strand synthesis system for reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen). PCR protocols were employed to amplify GFP cDNA. PCR amplification used two primers designed to amplify the entire coral GFP gene sequence. The primers are designated as GFP5' (5'ATTCCGCCCTGGTGATTTGG) and GFP3' (5'TTTTTCGGCTTCGATAACAAGT) and were designed on the basis of GFP sequence data from the coral *M. cavernosa* provided by Michael Matz (pers. comm.). The 50- $\mu\text{l}$

reaction mixture was composed of 2.5 units Taq DNA Polymerase (Applied Biosystems), 5  $\mu$ l 10 $\times$  PCR buffer with 15 mM MgCl<sub>2</sub>, 10 nmol each deoxynucleotide triphosphate (dNTP) (1.0  $\mu$ l of 10 mM), 12.5 nmol GFP5' primer (1.25  $\mu$ l of 10 mM), 37.5 nmol GFP3' primer (3.75  $\mu$ l of 10 mM), and 2  $\mu$ l ss cDNA. PCR amplifications were carried out in an Eppendorf Mastercycler Gradient 5331 (Eppendorf Scientific) with an initial denaturation step of 94°C for 2 min, one cycle of 95°C for 10 s, 50°C for 1 min, and 72°C for 40 s, followed by 35 cycles of 95°C for 10 s, 52°C for 30 s, and 72°C for 40 s.

The PCR product was visualized on a 1.5% TAE-agarose gel stained with ethidium bromide. Bands were excised from the gel and purified by using a Qiaquick gel extraction kit (Qiagen). DNA was eluted in milliQ water and quantified on a 2% agarose gel with a Low DNA mass ladder (Invitrogen). This product was TA cloned into competent JM109 *Escherichia coli* cells by means of the p-GEM T vector system (Promega). Small scale purification of plasmid DNA was completed with the Wizard Plus minipreps DNA purification system (Promega). Restriction analysis with the restriction enzyme *Bst*I was performed to confirm the presence and size of the cloned PCR product.

Plasmid DNA was prepared for dideoxynucleotide chain termination sequencing by using the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech). Direct sequencing of the double stranded PCR products was performed on an ABI Prism 377 DNA sequencer (Applied Biosystems). Cycle sequencing reactions were completed with SP6 and T7 promoter primers (Promega), as well as the primers used for initial PCR amplification, GFP5' and GFP3'. Nested primers were used in cycle sequencing as well: 393R (5'TCAGGAAACGTCTGCTTGA), 726R (5'TGGTCCACAAAGTGATAGTCTG), and degenerate primer PROB (5'CCTGCCRAYGGTCCNGTKATG) (Matz et al. 1999).

## Results

**Fluorescence microscopy**—Examination of sections of *M. cavernosa* from 3, 10, 18, 23, and 30 m by epifluorescence microscopy revealed that GFP was distributed almost exclusively within the epithelial cells with sporadic granules located in the gastrodermal cells where the zooxanthellae reside (Fig. 1). The cellular distribution was visually similar for samples of *M. cavernosa* at all depths of collection.

**Fluorescence properties**—Figure 2 shows excitation and emission spectra that represent the range of characteristic fluorescence responses measured to date from host tissues of a variety of Caribbean corals. In this paper, we concentrate on the pigment represented in Fig. 2B, with narrow-band emission in the green. The spectra illustrated in Fig. 2A are similar to the published excitation and emission spectra for amFP486 from an Indo-Pacific coral (Fig. 2, Matz et al. 1999), and this pigment from Caribbean species has been verified to be a GFP variant (unpubl. data). The spectra illustrated in Fig. 2A,B,D are common and can be found either singly or in combination in numerous species. When more than one is present, in some cases they are spatially colo-

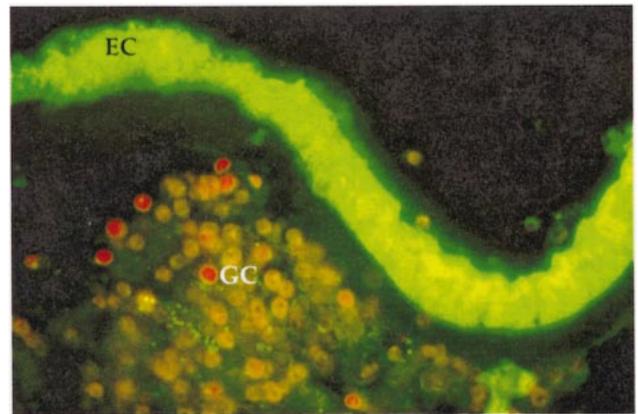


Fig. 1. Epifluorescence micrograph ( $\times 400$ ) of GFP localization in *M. cavernosa* from Horseshoe Reef (8 m) showing GFP primarily in the epithelial cells (EC) with granules located in gastrodermal cells (GC).

ated within the scale of the measurement, although the relative intensities may vary from spot to spot on a coral surface. In other cases, well-delineated patchy distributions of distinct fluorescence responses have been observed within a specimen. The spectra illustrated in Fig. 2C and Fig. 2E have so far only been observed in multiple specimens of a single species: the yellow fluorescence illustrated in Fig. 2C in *Agaricia humilis*, and the red fluorescence in Fig. 2E in one form of the widespread species *Porites astreoides*. In all cases the exact locations of the excitation and emission peaks can vary over plus or minus several nanometers in vivo. The two prominent excitation peaks for the 575 nm emission illustrated in Fig. 2D can vary greatly from specimen to specimen, with one or the other peak dominating to greater or lesser degree.

In situ and in vivo fluorescence emission spectra for the pigment illustrated in Fig. 2B reveal a sharp emission band in the green, with a peak at  $\sim 515$  nm and a spectral half band width of  $\sim 30$  nm. This can be found in numerous coral species (Table 1). This emission band can be populated by excitation from two absorption bands (Fig. 3), one centered at  $\sim 395$  nm (as in *L. cucullata*) and a second at  $\sim 504$  nm (as in *M. cavernosa*). The relative contributions of the two excitation bands to the emission signal are variable between and even within species. The location of the emission peak has been observed to vary over the range of 500 to 518 nm, with the excitation spectrum covarying, maintaining a nearly constant Stokes shift of 10 to 11 nm. Excitation/emission spectra of the purified tetrameric chromophore isolated from these two corals were identical to the excitation/emission spectra measured in intact corals (data not shown).

Analysis of fluorescence lifetimes from the laser-induced fluorescence kinetics (Fig. 4) indicated a bimodal decay with time constants of 1.9 and 3.0 ns and relative magnitudes of 14% and 86%, respectively. The amplitude weighted average lifetime was 2.85 ns. Lifetimes of green fluorescence measured in the corals *M. cavernosa* and *M. faveolata* by using the phase shift fluorometer were  $\sim 2.9$  to 3.0 ns. The observed lifetimes are similar to those of GFPs from the bioluminescent cnidarians *Aequorea* and *Renilla*, which range

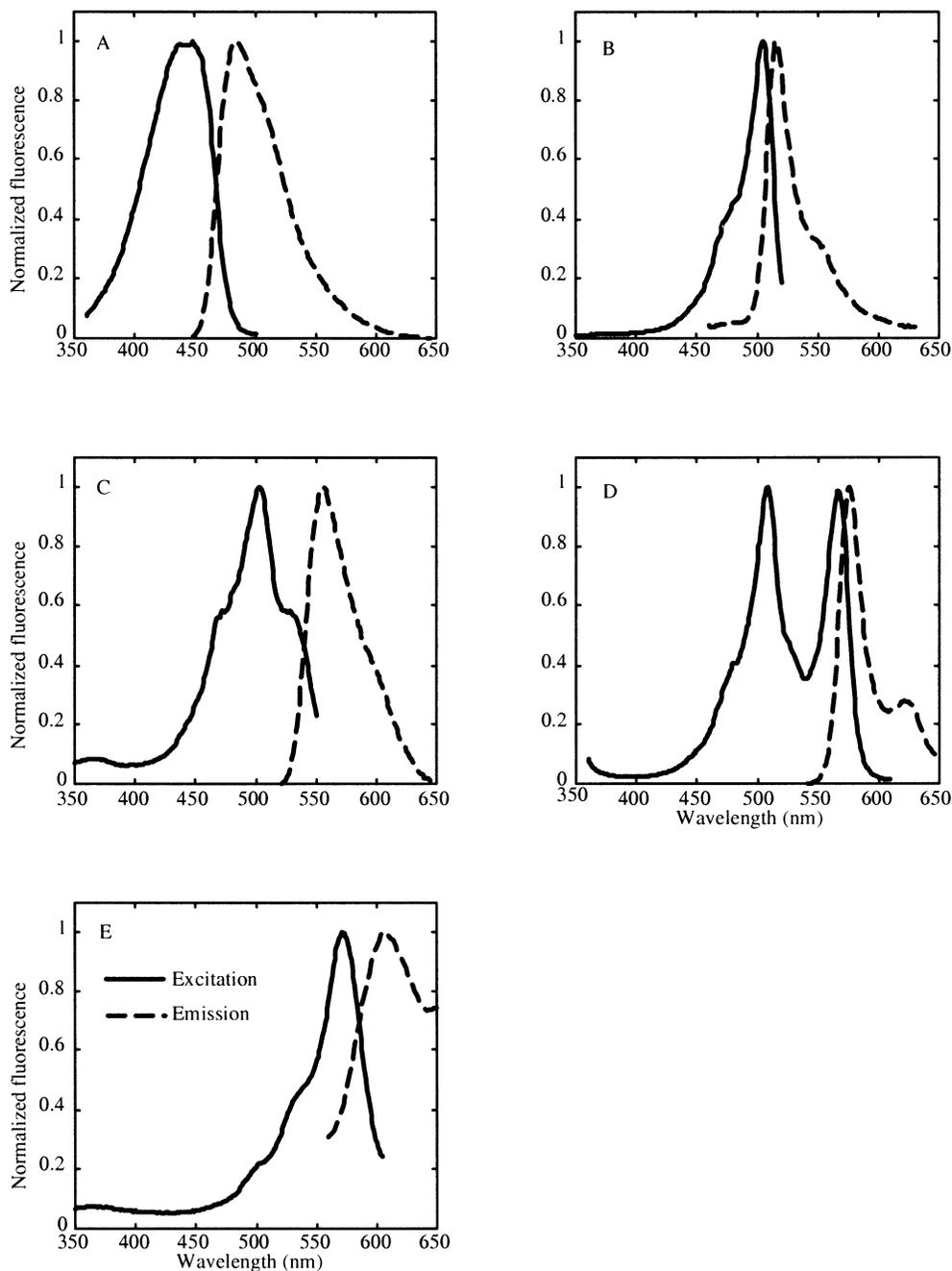


Fig. 2. Excitation and emission spectra that represent the range of characteristic fluorescence responses measured to date in host tissues of Caribbean corals. Measurement conditions: (A) *M. cavernosa*, excitation wavelength ( $\lambda_{\text{ex}}$ ) = 440 nm, emission wavelength ( $\lambda_{\text{em}}$ ) = 510 nm; (B) *P. astreoides*,  $\lambda_{\text{ex}}$  = 450 nm,  $\lambda_{\text{em}}$  = 530 nm; (C) *A. humilis*,  $\lambda_{\text{ex}}$  = 490 nm,  $\lambda_{\text{em}}$  = 565 nm; (D) *Scolymia* sp.,  $\lambda_{\text{ex}}$  = 520 nm,  $\lambda_{\text{em}}$  = 630 nm; (E) *P. astreoides*,  $\lambda_{\text{ex}}$  = 490 nm,  $\lambda_{\text{em}}$  = 620 nm.

from 2.8 to 3.3 ns (Perozzo et al. 1988; Swaminathan et al. 1997; Kneen et al. 1998). Based on the known extinction coefficient, spectral absorption bandwidth, and fluorescence lifetime, we estimate a fluorescence quantum yield of  $\sim 35\%$  for the chromophore in vitro.

In vivo excitation spectra for chlorophyll fluorescence were measured for dozens of coral specimens of a variety of species, with observable GFP fluorescence ranging from

weak or absent to intense. All of the spectra share the same general structure (Fig. 5), with excitation peaks corresponding to absorption of light by chlorophyll and accessory pigments such as peridinin. In none of these spectra was there any evidence of a peak in the excitation spectrum at wavelengths corresponding to absorption by GFP, which indicates the contribution of GFP fluorescence to photosynthesis is negligible. In a very few cases there was some indication of

Table 1. Caribbean corals examined for the presence of GFP by visual observation of ultraviolet-excited or blue-light-excited fluorescence, in situ or laboratory measurement of fluorescence emission spectrum, or Western blot analysis. The presence of GFP indicated by any of these approaches is indicated by an asterisk (\*). The absence of an asterisk indicates that the specimen was not examined by that method.

Species	Visual observation	Emission spectrum	Western blot
<i>Agaricia agaricites</i>	*	*	*
<i>Agaricia fragilis</i>			*
<i>Colpophyllia natans</i>	*	*	
<i>Dendrogyra cylindrus</i>		*	
<i>Isophyllastrea rigida</i>		*	
<i>Leptoseris cucullata</i>	*	*	*
<i>Madracis</i> sp.	*	*	
<i>Manicina areolata</i>	*	*	
<i>Meandrina meandrites</i>	*	*	
<i>Montastraea annularis</i>	*	*	
<i>Montastraea cavernosa</i>	*	*	*
<i>Montastraea faveolata</i>	*	*	*
<i>Montastraea franksii</i>	*	*	
<i>Mussa angulosa</i>	*	*	
<i>Mycetophyllia ferox</i>	*	*	
<i>Mycetophyllia lamarckiana</i>	*	*	
<i>Porites porites</i>		*	
<i>Porites astreoides</i>	*	*	*
<i>Scolymia</i> sp.	*	*	*

a decrease in chlorophyll excitation in the wavelength range of GFP excitation. The most extreme case of apparent photon removal by GFP in our data set was associated with an intensely green-fluorescent specimen of *Scolymia* sp., and is illustrated in Fig. 5, compared to a representative excitation spectrum for chlorophyll fluorescence measured from a non-

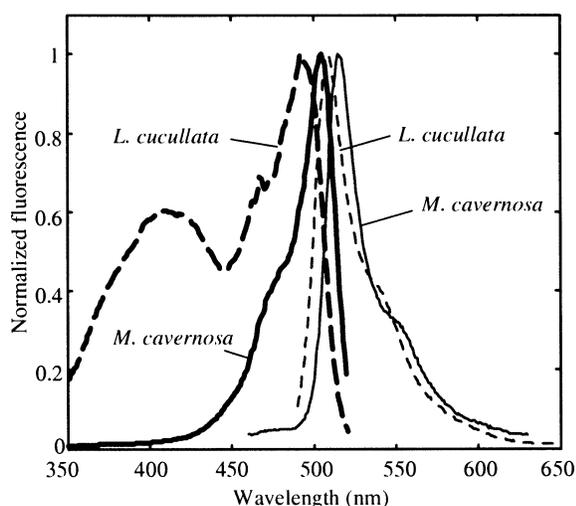


Fig. 3. Normalized fluorescence excitation and emission spectra for green fluorescence in live specimens of *M. cavernosa* (solid lines) and *L. cucullata* (dashed lines). Both excitation spectra were recorded for emission at 530 nm. The *M. cavernosa* emission was excited at 450 nm, while the *L. cucullata* emission was excited at 480 nm.

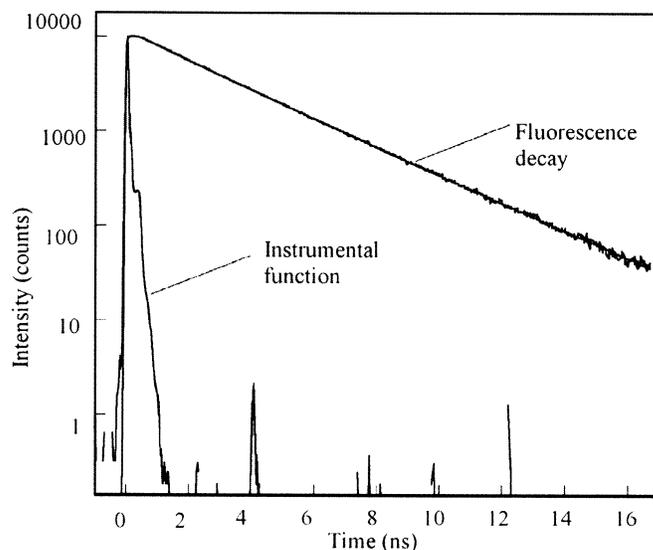


Fig. 4. Fluorescence decay kinetics for the GFP fluorescence from the coral *L. cucullata*. Fluorescence was excited at the wavelength of 410 nm and the emission was recorded at 510 nm.

fluorescent specimen of *M. cavernosa*. The excitation spectrum for the green fluorescence in the *Scolymia* specimen is indicated by the dotted line to show its correspondence with the spectral range of chlorophyll excitation decrease. The area under the chlorophyll excitation spectrum for the *Sco-*

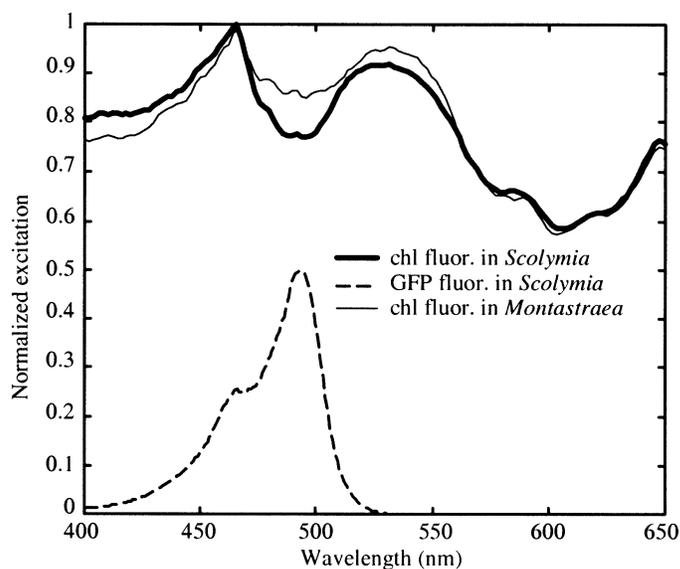


Fig. 5. Excitation spectra for chlorophyll fluorescence (emission at 690 nm) in a strongly green-fluorescent specimen of *Scolymia* sp. and a weakly fluorescent specimen of *M. cavernosa*. The decreased excitation in the 470 to 510 nm range may be due to absorption by GFP. The excitation spectrum for the green fluorescence in the *Scolymia* specimen is included (normalized to 0.5) to show its correspondence with the area of chlorophyll excitation decrease. Of dozens of such excitation spectra collected, this example from *Scolymia* is the only one that clearly indicated photon removal by GFP. No spectra showed any indication of enhancement of chlorophyll excitation by GFP.

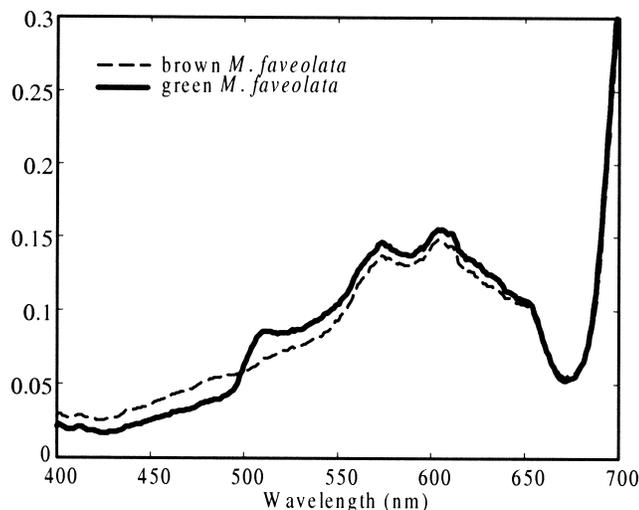


Fig. 6. Reflectance spectra of specimens of *M. faveolata* that appeared green and brown in ambient light. Both specimens were located at a depth of approximately 9 m at Horseshoe Reef. Both were oriented approximately horizontally, in exposed locations.

*lymia* specimen is approximately 0.7% less than that under the *M. cavernosa* spectrum, indicating, even in this extreme case, an insignificant effect on the overall absorption of photons for photosynthesis, assuming that the peak absorptions are approximately the same in both cases.

Reflectance spectra were measured in situ from several hundred coral specimens. Many contained strongly fluorescent GFP, as judged by their obvious green appearance in ambient light and by in situ fluorescence emission measurements, that covered the entire surface. Others exhibited only the brown appearance attributable to light absorption by zooxanthellae. Figure 6 shows reflectance spectra measured from two specimens of *M. faveolata* at a depth of 9 m at Horseshoe Reef. The spectrum measured from the green specimen shows an apparent reflectance peak at 510 nm that results from GFP fluorescence. Other than the increase in returned photons in this narrow spectral range, there is little difference between the spectra, and this was typical of many such measurements. The reflectance at wavelengths shorter than 500 nm is 1 to 2% lower in the green specimen than the brown in this case, but the variability in coral reflectance spectra is such that in some cases the reflectance from green specimens was a few percent greater than that of the brown in this region.

**Protein properties**—The isolated protein migrated on a HPLC size exclusion column with an apparent molecular weight of 100 kDa. SDS-PAGE of denatured proteins suggested that the complex is a homotetramer of  $24 \pm 2$  kDa polypeptides. The CD spectrum for GFP extracted from *L. cucullata* is nearly identical to that for pBAD recombinant GFP from *Aequorea* (Fig. 7). The characteristic  $\beta$ -sheet signal is observed in both spectra with a positive band at 195 nm and a negative band at 218 nm. A deconvolution analysis of the CD spectra for the two samples (Table 2) suggests that the secondary structure (and probably tertiary structure) of both proteins is very similar despite large differences in

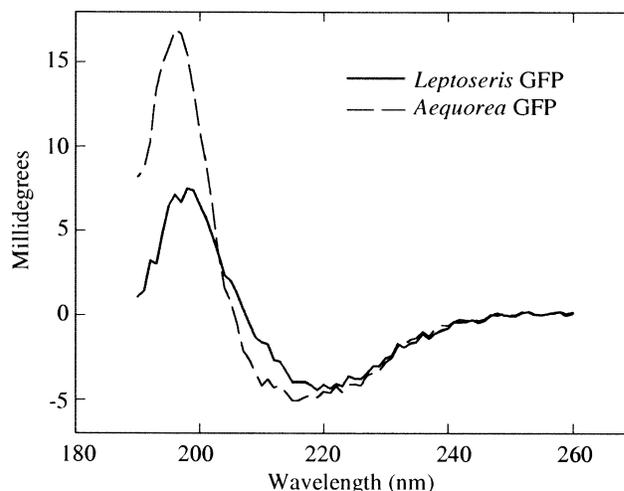


Fig. 7. Circular dichroism spectra of non-denatured *Leptoseris* GFP and pBAD recombinant GFP from *A. victoria*. The spectra are adjusted for approximately equal protein concentrations. The difference in the amplitude at 195 nm may reflect the attenuation of the optical rotation by the quaternary structure (tetrameric) in the native protein from the coral.

primary sequence of the N terminal. The antibodies produced against *Leptoseris* GFP did not crossreact with cloned *Aequorea* GFP.

**GFP distribution**—The fluorescence emission signature associated with GFP has been measured in situ in a number of scleractinian corals (Table 1). We have confirmed the presence of GFP in some of those species from the Bahamas using the polyclonal antibody to *Leptoseris* GFP described above. The bathymetric study of GFP protein concentration over the depth range of 3 to 30 m showed no significant differences for the effect of depth in either *M. faveolata* (ANOVA:  $P = 0.42$ ) or *M. cavernosa* (ANOVA:  $P = 0.64$ ) around LSI (Fig. 8A,B).

**GFP DNA sequences**—Only a single GFP homologous cDNA product was amplified and sequenced from either *M. cavernosa* (1046 bp) or *M. faveolata* (1046 bp). Untranslated regions (UTRs) are present on both the 5' and 3' ends of the sequence, and their functions are unknown. An alignment of the open reading frame (ORF) nucleotide sequences showed an overall homology with the coding region of *Aequorea victoria* GFP of 46.4% for both *M. cavernosa* and *M. faveolata*, while the two species of coral exhibited an

Table 2. Deconvolution analysis of CD spectra from 190 to 260 nm for *Leptoseris* GFP and a recombinant GFP from *Aequorea*.

	<i>Leptoseris</i> GFP	pBAD
Helix	6.7%	6.4%
Antiparallel	49.2%	50.0%
Parallel	3.9%	3.8%
Beta-turn	16.3%	16.5%
Random coil	28.8%	28.4%
Total sum	104.8%	105.1%

88.2% sequence homology when compared to each other (Fig. 9). The percent homology with *A. victoria* at the amino acid level was 31.1 and 28.4% for *M. cavernosa* and *M. faveolata*, respectively, and 82.7% with each other. The chromophore region of *A. victoria* is a three amino acid sequence, serine–tyrosine–glycine. In *M. cavernosa* and *M. faveolata*, the second and third positions of the chromophore are conserved while the first position shows a substitution of glutamine for serine in *M. cavernosa* and a substitution of aspartic acid for serine for *M. faveolata*. Substitutions in this first position are known to cause the loss of the excitation maximum in the ultraviolet portion of the spectrum and enhance overall fluorescence (Cubitt et al. 1995). The GenBank accession numbers are as follows: *M. faveolata* AF401282 and *M. cavernosa* AF384683.

## Discussion

The measurements and observations described here demonstrate that GFP is widely distributed among species and habitats in Caribbean scleractinian corals. While we have concentrated on scleractinian corals in this study, it is worth noting that the fluorescence spectral signature associated with GFP has also been measured in the corallimorpharian *Ricordea florida*, the zoanthids *Palythoa caribaeorum* and *Zoanthus pulchellus*, and the anemones *Condylactis gigantea* and *Phymanthus crucifer* (unpubl. data).

The present study does not answer the question of what function the presence of GFP in corals might serve, but it does provide evidence counter to several hypotheses that have been advanced in earlier work. It has been suggested in the past that coral host fluorescent pigments might function to (1) protect the corals and their endosymbionts from high levels of incident illumination (Kawaguti 1969; Salih et al. 2000), (2) provide additional photons for photosynthesis in light-limited situations (Schlichter and Fricke 1990), or (3) both, depending on the level of ambient illumination and the position of the fluorescent pigments in relation to the zooxanthellae (Salih et al. 1998). Our measurements of excitation spectra for chlorophyll fluorescence (Fig. 5) in specimens from a range of depths, with a wide range of intensities of GFP fluorescence, show no influence of GFP photon absorption either to enhance or reduce photosynthetic activity. Alternatively, it has been suggested (Salih et al. 2000) that GFP granules could contribute to a broadband removal of photons by reflection. Our measurements of reflectance (Fig. 6) from corals both with and without GFP widely distributed over the surface do not show any significant increase in reflectance attributable to the presence of the GFP. On the contrary, when the reflectance and fluorescence components of this apparent reflectance are separated (Fuchs 2001), a new reflectance minimum is revealed that corresponds to the peak in the absorption (excitation) spectrum of the GFP.

Corals have other mechanisms of photoprotection that are significantly more effective than that afforded by GFP. In zooxanthellae, as in most of the oxygenic photosynthetic organisms, excess excitation energy can be dissipated to heat via nonphotochemical quenching (for review see Falkowski

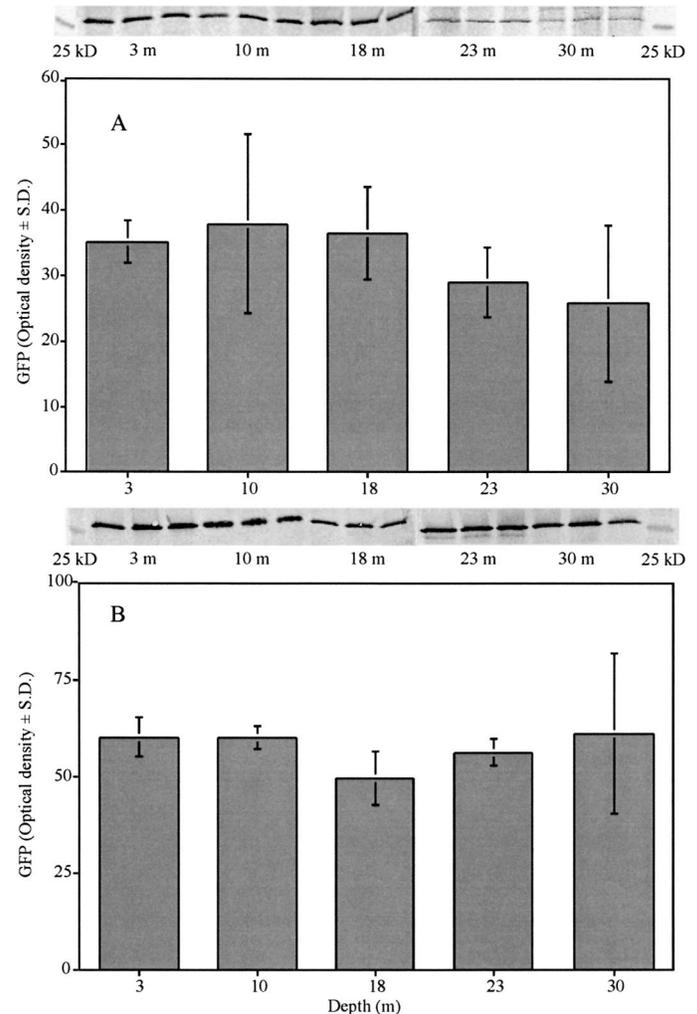


Fig. 8. Bathymetric sequence Western blots for *M. faveolata* and *M. cavernosa*. (A) Immunoblots and measured optical densities ( $\pm$ SD) for GFP from field samples ( $N = 3$  at each depth) of *M. faveolata*. (B) Immunoblots and measured optical densities ( $\pm$ SD) for GFP from field samples ( $N = 3$  at each depth) of *M. cavernosa*. No significant effect of depth was detected for either species.

and Raven 1997). In situ chlorophyll fluorescence measurements in corals showed that under supraoptimal irradiance, this process dissipates up to 80% of excess excitation energy, thereby providing efficient and highly dynamic photoprotection (Gorbunov et al. 2001). However, under low light (at depth or in shallow waters under cloudy skies), nonphotochemical quenching is virtually absent, maximizing the photosynthetic use of absorbed light energy (Gorbunov et al. 2001; Lesser and Gorbunov 2001). The capability of fluorescent pigments to protect zooxanthellae from excess light appears to be negligibly low compared with nonphotochemical quenching and likely has no physiological importance, at least in Caribbean corals.

If GFP played a role in photosynthesis, either enhancing it by adding photons or providing photoprotection by removing photons, we would expect to find a gradient of GFP concentration with depth or light level. This effect has been well demonstrated for the mycosporine-like amino acids

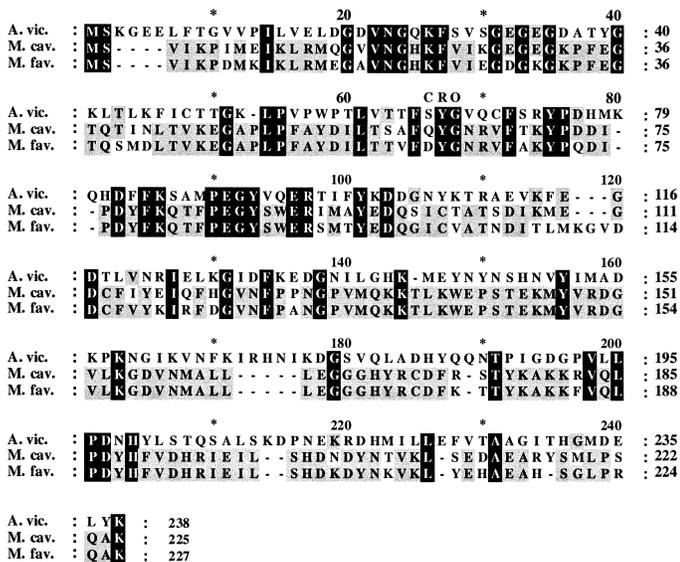


Fig. 9. Amino acid sequence comparison among GFPs from *A. victoria* (GenBank accession number: L29345), *M. cavernosa* (AF384683), and *M. faveolata* (AF401282). Amino acids shaded in black are conserved across all three species, those in gray are conserved between various pairwise comparisons. **CRO** indicates the three amino acid sequence of the chromophore.

(MAAs) that provide protection against ultraviolet radiation (Schick et al. 1996; Corredor et al. 2000) and for nonphotochemical quenching (Lesser and Gorbunov 2001). No significant trend was found, either positive or negative, for GFP concentration as a function of depth in either *M. faveolata* or *M. cavernosa*.

Another argument derives from the macroscopic spatial distribution of fluorescent pigments in corals. While in some cases the fluorescence appears distributed over the entire coral surface, in many cases bright fluorescence is localized to coral polyps or to structural features such as skeletal ridges. In such cases of constrained distribution the fluorescent pigments are typically found where the concentration of zooxanthellae is lowest. This physical segregation of the zooxanthellae and the fluorescent pigments would preclude the latter having a role in photosynthesis by either the addition or removal of photons.

During the work described here, we did not find any evidence of coral GFP coupling with a bioluminescent system. The absence of bioluminescence but presence of a GFP-like protein raises the question of the evolution and role of GFP in cnidarians. Recent molecular and morphological studies have clearly placed the Anthozoa as the basal class within the phylum Cnidaria (Bridge et al. 1995). The class Anthozoa contains not only the scleractinian corals, but also several species of *Renilla* and other luminescent pennatulaceans within the octocorallians. *Renilla reniformis* has a well-described bioluminescent system composed of a luciferin–luciferase complex coupled to GFP. Additionally, the other well-described luciferin–luciferase–GFP system comes from the hydromedusa *A. victoria* (in the class Hydrozoa and therefore a distant relative) and has also been characterized in other species from more derived classes of cnidarians (Morin 1974). Previous work has shown that luciferin–luciferase systems from a wide variety of taxa are not evolu-

tionarily conserved (Hastings 1983). For GFP, the available nucleotide sequencing information suggests a high degree of homology in the chromophore region, since the primary structure of the protein is critical to maintaining its fluorescent characteristics (Dopf and Horiagan 1996). Homology for other parts of the protein appears to be high among corals and probably accounts for the wide cross-reactivity of the GFP antibody made against *Leptoseria* GFP, but is considerably lower when compared to the “wild type” *A. victoria*. The most parsimonious interpretation of GFP evolution is that GFP did initially evolve in the basal cnidarian class, the Anthozoa. GFP was then coopted to improve the efficiency and emission characteristics of bioluminescent systems that subsequently evolved independently and multiple times in several more derived classes in the phylum Cnidaria. If this is true then GFP has very likely been present in scleractinian corals since the appearance of zooxanthellate taxa in the Tertiary period (Veron 1995), or possibly evolved as early as the suggested ancestor of corals, the Corallimorpharia (Stanley and Fautin 2001), from which GFPs have also been isolated (Matz et al. 1999). Although GFPs are also found within the more derived Hydrozoa (e.g., *Aequorea victoria*) attempts at isolating GFP from true jellyfish (Cnidaria: Scyphozoa) from the Phylum Ctenophora have failed to date (M. Lesser, unpubl.). Recent protein phylogenies of GFP (Labas et al. 2002) within the Anthozoa found no general congruence between GFP phylogeny and the systematic position of the host but did find that most GFPs from the Scleractinia and the Corallimorpharia fell within a single protein clade. For corals, fluorescent proteins and their non-fluorescent homologs probably radiated from their corallimorpharian ancestor(s) into the modern scleractinian taxa we see today.

This diversity and conservation of fluorescent proteins is likely dependent upon selective pressures associated with the habitat of modern zooxanthellate corals, but there is limited evidence to date on the specific role of GFP. Our spectral measurements and measurements of GFP concentration as a function of depth argue against a role in either photoprotection or enhancement of photosynthesis by the direct removal or addition of photons within the visible portion of the spectrum. The general absence of a strong excitation peak in the ultraviolet portion of the spectrum (290–400 nm) argues against a role in protecting against ultraviolet radiation (UVR), a function that is performed by MAAs (Shick et al. 1996). The same conclusion was reached by Gleason (1993) for the scleractinian coral *P. astreoides*, for which UVR resistance of the green morph was associated with increased production of MAAs and not with absorption by GFP. In some cases the green fluorescence of GFP is visible under ambient daylight illumination (Mazel and Fuchs 2002), and it is possible that this impact on color and visual contrast is the reason for the protein’s presence, although the ecological benefit to the coral of such color is uncertain (Wicksten 1989).

It remains possible that GFP plays a role in a coral’s ability to cope with environmental stresses such as elevated irradiances of either ultraviolet or visible radiation and elevated temperature, but through not yet understood physiological processes. Salih et al. (2000) observed a correlation between resistance to bleaching and the concentration of fluorescent pigments, but this was circumstantial and

the specific mechanism by which the resistance might have been afforded was not demonstrated. Recent results (Lesser unpubl. data) show an inverse relationship between the concentration of GFP and superoxide dismutase (SOD) proteins in host tissues of corals that had been subjected to solar radiation and temperature manipulations that resulted in the production of reactive oxygen species (ROS). SOD protects against superoxide radicals (Lesser 1996, 1997), and perhaps GFP is involved in the nonenzymatic scavenging of ROS in the hyperoxic environment of corals. Much of the current work with GFP is motivated by its value as a reporter molecule for studies requiring an understanding of transcriptional regulation, but much remains to be done to understand its role in coral physiology and ecology.

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