

Spectral Diversity of Fluorescent Proteins from the Anthozoan *Corynactis californica*

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Abstract Color morphs of the temperate, nonsymbiotic corallimorpharian *Corynactis californica* show variation in pigment pattern and coloring. We collected seven distinct color morphs of *C. californica* from subtidal locations in Monterey Bay, California, and found that tissue- and color-morph-specific expression of at least six different genes is responsible for this variation. Each morph contains at least

three to four distinct genetic loci that code for these colors, and one morph contains at least five loci. These genes encode a subfamily of new GFP-like proteins, which fluoresce across the visible spectrum from green to red, while sharing between 75% to 89% pairwise amino-acid identity. Biophysical characterization reveals interesting spectral properties, including a bright yellow protein, an orange protein, and a red protein exhibiting a “fluorescent timer” phenotype. Phylogenetic analysis indicates that the FP genes from this species evolved together but that diversification of anthozoan fluorescent proteins has taken place outside of phylogenetic constraints, especially within the Corallimorpharia. The discovery of more examples of fluorescent proteins in a non-bioluminescent, nonsymbiotic anthozoan highlights possibilities of adaptive ecological significance unrelated to light regulation for algal symbionts. The patterns and colors of fluorescent proteins in *C. californica* and similar species may hold meaning for organisms that possess the visual pigments to distinguish them.

Christine E. Schnitzler and Robert J. Keenan contributed equally to this work.

Data deposition footnote: The GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>) accession numbers for the genes and gene products discussed in this paper are: ccalRFP1 (AY823226); ccalYFP1 (AY823227); ccalRFP2 (DQ065851); ccalGFP1 (DQ065852); ccalOFP1 (DQ065853); ccalGFP3 (DQ899732)

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Introduction

Organisms can respond to changing external variables by altering their morphology or behavior (Meroz-Fine et al. 2003; Relyea 2004). Morphological responses in marine invertebrates include changing the color or pattern of pigmentation or adjusting pigmentation levels (Wicksten 1989; Tupen 1999). Such pigment modification can be achieved on an evolutionary timescale by inherited genetic differences (polymorphisms) arising from mutation and natural selection, or within an organism’s lifetime by

exhibiting phenotypic plasticity in pigment expression in response to environmental factors (Hansson 2004). Among morphs of a particular species, differences in pigmentation can be attributed to patterns of inheritance and expression encoded by multiple loci or to variations in sequence encoded by different alleles at the same locus (Kelmanson and Matz 2003). However, as studies of the diversity of color patterns on butterfly wings have shown (Brakefield and French 1999), the origin of differences in pattern or color of pigmentation can be quite complex and may evolve from some combination of both genes and environment. In many cases, factors influencing pigmentation differences among morphs of the same species are unknown, and even basic analyses of the pigments involved have not been attempted (Wicksten 1989).

Recent studies have shown that pigmentation in many anthozoan species is the result of proteins from the green-fluorescent protein (GFP) family (Matz et al. 1999; Dove et al. 2001; Labas et al. 2002; Kelmanson and Matz 2003; Bessette and Daugherty 2004; Wiedenmann et al. 2004). The synthesis of the chromophore of these proteins is unique among pigment molecules because it is achieved autocatalytically in the presence of molecular oxygen, making these proteins useful biological markers (Zhang et al. 2002; Verkhusha and Lukyanov 2004; Shaner et al. 2005). Beyond their broad impact as tools for biomedical research, fluorescent proteins (FPs) are ideal subjects for ecologists interested in the functional adaptations of fluorescence, and for evolutionary biologists interested in protein and color evolution. Because emission colors of individual proteins can be determined by recombinant expression in bacterial systems, it is possible to conduct phylogenetic studies of color evolution when sequence data are available (Matz et al. 2002; Ugalde et al. 2004).

The Corallimorpharia is the smallest extant order within the anthozoan subclass Zoantharia (Hexacorallia), containing approximately 40 species distributed worldwide in tropical and temperate waters (Carlgren 1949; Dunn 1982). Morphologically, corallimorpharians are most similar to stony corals (scleractinians), although, like sea anemones (actinarians), they lack a calcareous endoskeleton. A recent study analyzing whole mitochondrial genomes placed corallimorpharians within the Scleractinia (Medina et al. 2006). Although there were difficulties in obtaining the full gene complement for *C. californica* in the study, this species was considered basal to other corallimorpharians, including *Discosoma* and *Ricordea*, tropical genera whose fluorescent proteins are known. DsRed, one of the first red fluorescent proteins, was cloned from a species of *Discosoma* (Matz et al. 1999).

The temperate, nonsymbiotic, and non-bioluminescent corallimorph *Corynactis californica* (Carlgren 1949) is an ideal candidate for the investigation of pigmentation. *C. californica* exhibits a variety of color morphs, including

shades of lavender, pink, orange, brown, and red (West 1979). These morphs, in turn, exhibit patterns of fluorescent pigmentation under blue-light excitation.

C. californica lives from the lower intertidal zone to at least 50 meters (Chadwick 1987) and can be found from Washington, USA, to central Baja California, Mexico. This and other “strawberry anemones” are found throughout the temperate oceans of the world, often occurring in areas with little or no direct sunlight, underneath rock ledges or in caves (Hand 1954), or largely covered by macroalgae (C.E. S., pers. obs.). *C. californica* reproduces sexually by annual spawning and asexually by longitudinal fission (Chadwick and Adams 1991; Holts and Beauchamp 1993), and it commonly occurs as large aggregations consisting of identical asexually-produced clones exhibiting the same pigmentation (Haderlie et al. 1980). Copepods, nauplius larvae, and other small animals comprise the diet of this active suspension feeder (Haderlie et al. 1980).

Through cloning and expression, we describe the molecular basis for coloration in *Corynactis californica*. By comparing the fluorescent pigmentation in seven color morphs of *C. californica* at both the whole animal (in vivo) and molecular (in vitro) levels, we demonstrate tissue-specific expression patterns within and between morphs, and discuss these variations in relation to their possible ecologic role.

Materials and Methods

Sample Collection

Multiple polyps from individual clones of seven distinct color morphs of *C. californica* (designated Lavender, Pink1, Pink2, Red, Orange1, Orange2, and Orange3) were collected with hand tools, using SCUBA for subtidal specimens, from three locations in Monterey Bay, CA (California Scientific Collecting Permit 803069). Specimens were kept alive in a 14.5°C running seawater lab at the Monterey Bay Aquarium Research Institute (MBARI).

In Vivo Fluorescence Emission Spectra Measurements

Polyps were examined for fluorescence under a Nikon SMZ-1500 epifluorescence microscope with long-pass emission filters and excitation at 410 nm, 470 nm, and 500 nm. A Nikon Coolpix 5000 digital camera attached to the microscope was used to document images of whole animal (in vivo) fluorescence. All fluorescence images were taken under blue light (470 nm) excitation. Fluorescence emission spectra of live specimens were collected with a USB-2000 fiber-optic spectrometer (Ocean Optics, Dunedin, FL) under blue light excitation. Tissue-specific emission spectra from five tissue types (mouth, oral disc,

base of tentacles, tentacles, and body column) were measured by focusing the microscope on a single type of tissue of an individual polyp (N=5), then holding the fiber optic up to the eyepiece of the microscope to record the spectrum through the microscope.

FACS-Based Expression Cloning of ccalRFP1 and ccalYFP1

Total RNA was isolated from a single red *Corynactis californica* polyp using TRIzol Reagent (Invitrogen, Carlsbad, CA) as per manufacturer's instructions and then treated with DNase I (Invitrogen) to remove contaminating genomic DNA (Simms et al. 1993). A cDNA library was constructed using a SMART cDNA synthesis kit (BD Biosciences Clontech, Palo Alto, CA), ligated into pTriplEx2 (BD Biosciences Clontech), transformed into electrocompetent TG1 cells (Stratagene, La Jolla, CA), plated on LB/agar supplemented with 50 µg/ml carbenicillin (carb) and incubated at 37°C overnight to obtain a library of approximately 1×10^6 unique clones. Colonies scraped from the transformation plates were combined and diluted into 50 ml LB/carb liquid culture supplemented with 1.0 mM IPTG. At 4, 8, and 16 h post-induction, a small aliquot of culture was subjected to FACS screening with a MoFlo cytometer (DakoCytomation, Carpinteria, CA). The following excitation/emission wavelengths were monitored simultaneously during screening: 407 nm/510 to 550 nm; 488 nm/510 to 550 nm, 560 to 600 nm, 615 to 645 nm, and 655 to 685 nm. From almost 10^7 events, 400 positive events were sorted directly onto LB/agar plates supplemented with 50 µg/ml carb and 1.0 mM IPTG and grown overnight at 37°C. Plasmid DNA was prepared from 168 of these clones and subjected to automated DNA sequencing (ABI 4500, Applied Biosystems, Foster City, CA). Two intact ORFs, corresponding to ccalRFP1 and ccalYFP1, were obtained using this strategy.

PCR-Based Cloning of ccalRFP2, ccalOFP, ccalGFP1, and ccalGFP3

RNA Isolation and Degenerate RT-PCR Total RNA was isolated from single whole polyps of each of the seven color morphs that was used for spectral measurement with a RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instruction, treated with DNase I (Invitrogen), and stored at -80°C until needed. cDNA was synthesized from total RNA via reverse transcription with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen).

A set of degenerate primers based on the ccalYFP1 and ccalRFP1 sequences was designed to amplify additional fragments of genes coding for related FPs in this species. Primers were designed to amplify the internal portion of the gene and 2 of 47 nucleotides in this primer pair were degenerate.

Degenerate PCR reactions for each color morph cDNA consisted of 2 µl cDNA, 2 U Platinum *Taq* polymerase (Invitrogen), 1x Platinum *Taq* buffer, 0.2 µM of each degenerate primer, and 200 µM dNTPs. Cycling parameters were: 94°C for 2 min, 35 cycles of 94°C for 15 s, 62°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. Resulting PCR products were gel purified (QIAQuick Gel Extraction Kit, Qiagen), cloned into a TOPO TA cloning vector (pCR2.1-TOPO, Invitrogen), and then sequenced. Two unique cDNA fragments (named ccalRFP2 and ccalOFP1) with sequence homology to ccalYFP1 and ccalRFP1 resulted from this approach and were selected for further study. A second set of degenerate primers based on the ccalYFP1, ccalRFP1, ccalRFP2, and ccalOFP1 sequences was designed after the full-length coding regions of the two new genes were determined (see "Determining cDNA Ends"). These primers were designed to amplify the internal portion of the gene, and 13 of 48 nucleotides in this primer pair were degenerate. Using this second set of primers against cDNA, and an annealing temperature of 50°C, two additional fragments (named ccalGFP1 and ccalGFP3) homologous to other FP genes from *Corynactis californica* were cloned.

Determining cDNA Ends The sequence of the 5' and 3' ends of the four new gene fragments was obtained using either rapid amplification of cDNA ends using a SMART RACE cDNA Amplification Kit (BD Biosciences Clontech), or degenerate primers directed against a non-coding region adjacent to the coding region of ccalYFP1 and ccalRFP1 obtained from the library screening and paired with gene-specific primers designed from each new fragment.

Full Length Cloning Once full-length sequence information of all six FPs was known, the complete coding regions were amplified via PCR from a single color morph cDNA using gene-specific primers designed for each FP gene and PfuUltra Hotstart polymerase (Stratagene), a high-fidelity, proofreading polymerase. These products were cloned and sequenced.

PCR Survey of FPs in the Different Color Morphs

Degenerate primers and gene-specific primers designed to unambiguously amplify each of five FPs were directed against cDNA from all seven color morphs of *Corynactis californica* to determine the presence of each gene in each morph. CcalGFP3 was not included in this survey.

Protein Production

Expression of ccalGFP3, ccalYFP1, ccalOFP1, and ccalRFP2 was performed in *E. coli* NovaBlue/pREP4 cells transformed with a modified pQE31 vector containing a

tobacco etch virus (TEV) protease cleavage site inserted between the N-terminal hexahis-tag and the polylinker. Typically, a 10 ml saturated overnight culture was used to inoculate 1L LB culture (in a 2.8L baffled Fernbach flask) supplemented with ampicillin, kanamycin and 0.1 mM IPTG. Cells were grown overnight (ccalYFP1, ccalRFP2) or for approximately four days (ccalGFP3 and ccalOFP1) at room temperature, and then pelleted by centrifugation.

Expression of the green and red forms of ccalRFP1 (see “Maturation” below) was performed in *E. coli* BL21(DE3) cells transformed with a modified pET28 vector containing a TEV protease cleavage site inserted between the N-terminal hexahis-tag and the polylinker. To produce the green form of ccalRFP1, a 5 ml saturated overnight culture was used to inoculate a 250 ml LB culture (supplemented with kanamycin and chloramphenicol) in a 1 L baffled flask. After the culture reached an OD₆₀₀ of ~0.6, the cells were induced with 0.1 mM IPTG. After 2 days at 30°C, most of the protein was found in the culture medium; cells and cellular debris were discarded, and ccalRFP1 was purified directly from the culture medium as described below. To produce the red form of ccalRFP1, a 5 ml saturated overnight culture was used to inoculate a 250 ml LB culture (supplemented with kanamycin and chloramphenicol) in a 1 L baffled flask. After growing the culture to an OD₆₀₀ of ~0.6, the cells were transferred to a 500 ml non-baffled flask and induced with 0.1 mM IPTG. After 3 h at 30°C, the cells were harvested by centrifugation.

Cell pellets were resuspended in BPER lysis buffer (Pierce, Rockford, IL) supplemented with 500 mM NaCl, 1 mg/ml lysozyme, 20 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride and sonicated. The lysate supernatant was purified by Ni-NTA agarose column chromatography (Qiagen), and bound protein was eluted with 20 mM HEPES pH 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol and 200 mM imidazole. For purification of the green form of ccalRFP1, the culture medium was applied directly to the Ni-NTA column. Purified proteins were buffer exchanged into 20 mM HEPES, pH 8.0, 500 mM NaCl, and 1 mM DTT, concentrated to ~5 to 10 mg/ml using Amicon Ultra centrifugal filter units (Millipore, Billerica, MA), and stored at -80°C.

Spectroscopic Analysis

Absorbance measurements and spectra were collected with a Shimadzu UV-1650PC spectrophotometer. Fluorescence excitation and emission spectra were collected on a HORIBA Jobin Yvon Fluoromax-3 spectrofluorometer, and an OceanOptics USB2000 spectrometer. Unless otherwise noted, all spectroscopic measurements were made with purified His-tagged protein in 20 mM HEPES, pH 8.0, 500 mM NaCl, and 1 mM DTT.

Protein concentrations were determined by the alkali denaturation method (Gross et al. 2000; Ward 2005), using the absorbance of the chromophore after denaturation in 0.1 N NaOH ($\epsilon_M=44,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 447 nm) to estimate the molar extinction coefficient of the native protein at the visible absorbance maximum. Care was taken to ensure complete protein denaturation without degradation of the 447 nm-absorbing species during the course of the measurement.

Quantum yields were determined using the comparative method as described previously (Lakowicz 1999; Baird et al. 2000). CcalGFP3, ccalYFP1, ccalOFP1 and ccalRFP1 (green form) were excited at 480 nm, and EGFP (0.60; Patterson et al. 2001) and fluorescein (0.92 in 0.1N NaOH; Magde et al. 2002) were used as standards. CcalRFP1 (red form) and ccalRFP2 were excited at 500 nm, and DsRed (0.79; Shaner et al. 2004) and rhodamine B (0.70 in EtOH; Arbeloa et al. 1989) were used as standards.

Maturation experiments on ccalRFP1 were conducted using a Roper Scientific back-illuminated CCD camera mounted on an Acton/Princeton Instruments SpectraPro monochromator. Rapidly purified protein was placed in a small-volume quartz cuvette and monitored uncovered in the dark every 20 min for 16 h. Stimulation was a 1-s pulse from a 470-nm-emitting LED, which was off except during the brief measurement interval.

Sequence and Phylogenetic Analysis

Phylogeny of the *Corynactis californica* fluorescent proteins was estimated relative to other anthozoan FPs using maximum likelihood, parsimony, and Bayesian methods. First, an amino acid alignment was generated with T-Coffee (Notredame et al. 2000) or MUSCLE (Edgar 2004), and adjusted using MacClade (Maddison and Maddison 2000). Variable-length tails and any regions that could not be unambiguously aligned were masked from subsequent analyses. To choose an appropriate set of assumptions, the program MODELGENERATOR (Keane et al. 2006) applied AIC1, AIC2, and BIC2 metrics to a variety of possible substitution matrices and rate assumptions (Akaike 1974). The results from AIC2 and BIC2, which are more conservative with regard to over-parameterization, indicated the appropriate model was WAG+Γ, with eight rate categories. All three metrics favored WAG-based models (Whelan and Goldman 2001), with variants of the JTT model (Jones et al. 1994) as secondary choices. We performed our analyses using both models, without substantial disagreement between the resulting topologies.

For maximum likelihood using PhyML (Guindon and Gascuel 2003), we ran 100 bootstraps using 8 gamma rate categories, P_{inv} and alpha estimated based on the data matrix. For Bayesian analyses, we ran the multiprocessor version of MrBayes 3.04 (Huelsenbeck and Ronquist

2001), within the MPI package installed on a Quad G5 Macintosh. We used the default settings except for selecting the fixed WAG model, and running four chains for 2,000,000 generations, sampling every 100 generations. Convergence diagnostics, examined with the help of AWTY (Wilgenbusch et al. 2004), indicated a conservative burn-in period of 250,000. The runs all reached stationarity, and adjusting the temperature parameter (from 0.2 from 0.1) or the burn-in, did not noticeably affect the topology, swap rate, or other indices of convergence. Parsimony analysis and bootstraps were conducted using PAUP* 4.0b10 for MacOSX/Unix (Swofford 2003).

The most likely tree, annotated with consensus support values, was arranged and visualized using ATV (Zmasek and Eddy 2001) and FigTree (Rambaut 2007). Trees were rerooted with Phyutility 1.1a (Smith 2007) if needed, and then annotated manually using Adobe Illustrator. Nodes with bootstrap support between 50% and 90% and those with >90% support were annotated symbolically on the tree.

Results

In Vivo Fluorescence Emission Spectra Measurements

Fluorescence was compared for seven color morphs of *Corynactis californica* collected from the surface to 30 m depth in Monterey Bay, CA. Differences in color and tissue localization coincided with distinctive patterns of fluorescence, visible in paired images of the color morphs under white and blue light (Fig. 1). Two general patterns of

fluorescence are apparent: Pattern A is characterized by a single major emission in all tissues (Pink2, Orange2, Orange1, and Red morphs; Fig. 1h–k); Pattern B is characterized by bright fluorescence emission at the base of the tentacles and around the mouth, but little fluorescent emission in other tissues (Lavender, Pink1 and Orange 3 morphs; Fig. 1l–n). To investigate these patterns in more detail, we measured fluorescence emission spectra from five tissue types (mouth, oral disc, base of tentacles, tentacles and body column) for each color morph (averages in Table 1). Fluorescence emission was detected in all tissues of all morphs, although some tissues also have nonfluorescent absorptive pigments. Overall, emission peaks centered around three wavelengths (518, 563, and 599 nm). Most morphs had multiple emission peaks for each tissue type with a single peak dominating (data not shown), indicating a mixture of pigments colocalized in individual tissues.

The major emission peaks for a given Pattern A color morph were the same in all tissue types observed. For example, the Red, Orange1 and Pink2 morphs had a dominant emission peak around 600 nm in all tissues, while the Orange2 morph had a major emission peak around 562 nm in all tissues. In contrast, for any given Pattern B morph, different major emission peaks were observed for different tissues. For example, the major emission peak at the base of the tentacles in the Lavender morph was centered around 562 nm, while the mouth showed a major emission peak around 597 nm. Taken together, these data indicate that similar mixtures of fluorescent pigments are present in all *C. californica* color

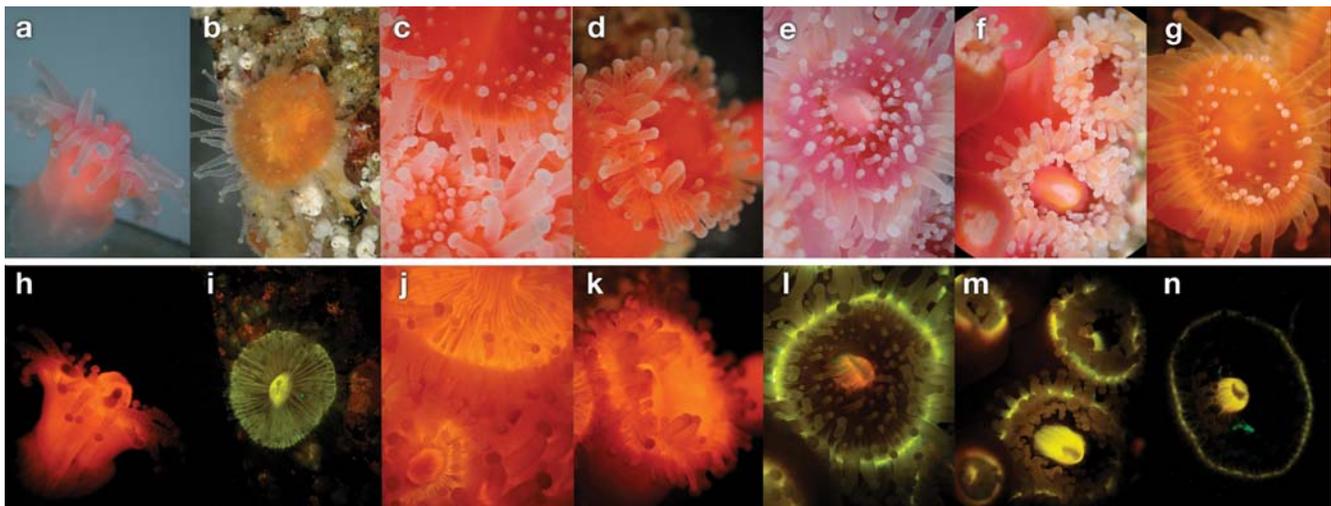


Fig. 1 Paired images of brightfield (top row) and blue-illumination (bottom row) of seven *C. californica* color morphs. Pink2 morph, panels **a, h**; Orange2, panels **b, i**; Orange1, panels **c, j**; Red, panels **d, k**; Lavender, panels **e, l**; Pink1, panels **f, m**; Orange3, panels **g, n**

Table 1 In vivo analysis of fluorescence in *C. californica* color morphs

Color Morph	Emission Maxima (nm)			<i>C. californica</i> FP gene						Class
				GFP1	GFP3	YFP1	OFP1	RFP1	RFP2	
Pink2	599	–	517	–	N.D.	+	+	+	+	A
Orange2	–	562	520	–	N.D.	+	+	+	+	
Orange1	599	562	520	–	N.D.	+	+	+	–	
Red	601	–	516	–	N.D.	+	+	+	–	B
Lavender	596	563	517	+	+	–	+	+	+	
Pink1	598	564	517	+	N.D.	–	+	+	+	
Orange3	–	564	518	+	N.D.	–	+	+	+	

Mean fluorescence emission wavelength (λ_{max}) measured in tissues from each of the seven *C. californica* color morphs; standard deviations (for N = 25; 5 tissue types from each of 5 polyps) are within ± 0.5 to 4.4 nm in all cases. Major emission peaks are shown in bold; minor peaks are in plain. The presence (+) or absence (–) of each FP gene also is indicated for each color morph. *ND* not determined

morphs, but that they are often expressed in a tissue-specific manner.

FACS and PCR-Based Cloning of ccalFPs

To identify putative *C. californica* fluorescent proteins we prepared a cDNA library from a single red polyp and screened it for expression in *E. coli* by FACS. Two intact open-reading frames encoding new GFP homologs were isolated. These genes encode 221 residue proteins, designated ccalYFP1 and ccalRFP1, according to their in vitro fluorescent properties (described below). In the initial round of PCR with degenerate primers, two additional unique FP gene fragments, termed ccalRFP2 and ccalOFP1, were identified, and these sequences were aligned with ccalYFP1 and ccalRFP1 to design new degenerate primers. A second PCR screen using these new primers resulted in the identification of two additional gene fragments, called ccalGFP1 and ccalGFP3. The full-length ccalRFP2 and ccalOFP1 genes encode 226 residue proteins, while ccalGFP1 and ccalGFP3 encode 221 residue proteins.

PCR Survey of ccalFPs in Color Morphs

Degenerate and gene-specific primers were used to perform a PCR survey of the cDNA generated from the seven *C. californica* color morphs. Presence or absence of a particular gene is noted in Table 1. Each of the color morphs contain all but one or two of the ccalFP genes. CcalGFP3, which was originally cloned from the Lavender morph, was not included in this distributional survey.

Spectral and Biochemical Properties

To characterize the spectral and biochemical properties of the *C. californica* fluorescent proteins in vitro, we subcloned them with a cleavable N-terminal hexahistidine tag for over-expression in *E. coli*. Although it accumulates to high levels in *E. coli*, ccalGFP1 is insoluble, and attempts to refold it were unsuccessful. Preliminary characterization by size exclusion chromatography indicates that the *C. californica* FPs share a tendency to oligomerize into dimers and/or tetramers in solution (data not shown).

Table 2 In vitro properties of *C. californica* fluorescent proteins

<i>C. californica</i> FP	Absorbance maximum (nm)	Emission maximum (nm)	Extinction coefficient ($M^{-1} cm^{-1}$)	Fluorescence quantum yield	Brightness ($mM^{-1} cm^{-1}$)
GFP3	505	517	144000	0.48	69
YFP1	514	523	142000	0.75	107
OFP1	508/551	561	85000	0.52	44
RFP1 green	504	517	78000	0.46	36
RFP1 red	568	598	92000	0.59	54
RFP2	504/558	516/587	61000	0.05	3
EGFP	488	507	56000	0.60	34
DsRed	558	583	75000	0.79	59

The corresponding values for EGFP (Patterson et al. 1997) and DsRed (from Shaner et al. 2004) are shown for comparison

The absorbance and emission spectra for the purified *C. californica* fluorescent proteins are shown in Fig. 3. The corresponding spectral properties are summarized in Table 2. The fluorescence spectra of the purified proteins are generally representative of the fluorescence measured in vivo (Table 1), with each of the main color classes represented: green (ccalGFP1, ccalGFP3), yellow (ccalYFP1), orange (ccalOFP1) and red (ccalRFP1, ccalRFP2). The absorbance spectra of ccalGFP3 and ccalYFP1 suggest the presence of a GFP-like chromophore, with an absorbance maximum at 505 nm (ccalGFP3) and 515 nm (ccalYFP1). These are the brightest of the *Corynactis* FPs; ccalYFP1 is more than three times brighter than EGFP, and almost twice as bright as DsRed. In contrast, ccalRFP2 is only marginally fluorescent at neutral pH and might well be classified a chromoprotein. The absorbance spectrum of ccalOFP1 reveals two chromophore species, one with an absorbance maximum of 508 nm, and the other with an absorbance

maximum at 551 nm. The relative ratio of the 508 and 551 nm absorbing species seems to be relatively insensitive to the method used to express and purify the protein. Efficient FRET between the 508 nm- and 551 nm- absorbing chromophore species in the ccalOFP1 dimer/tetramer results in a bright orange fluorescence at 561 nm following excitation at 480 nm.

Maturation of ccalRFP1

Expression and purification of ccalRFP1 typically resulted in a protein that matured to a stable end point containing varying amounts of green- and red-emitting species. Certain preparations, however, exhibited a striking transition from an initial green to a stable bright red endpoint after overnight incubation. We tested various growth and maturation conditions, including pH, salt, protein concentration, temperature, time, expression strain, and light, but

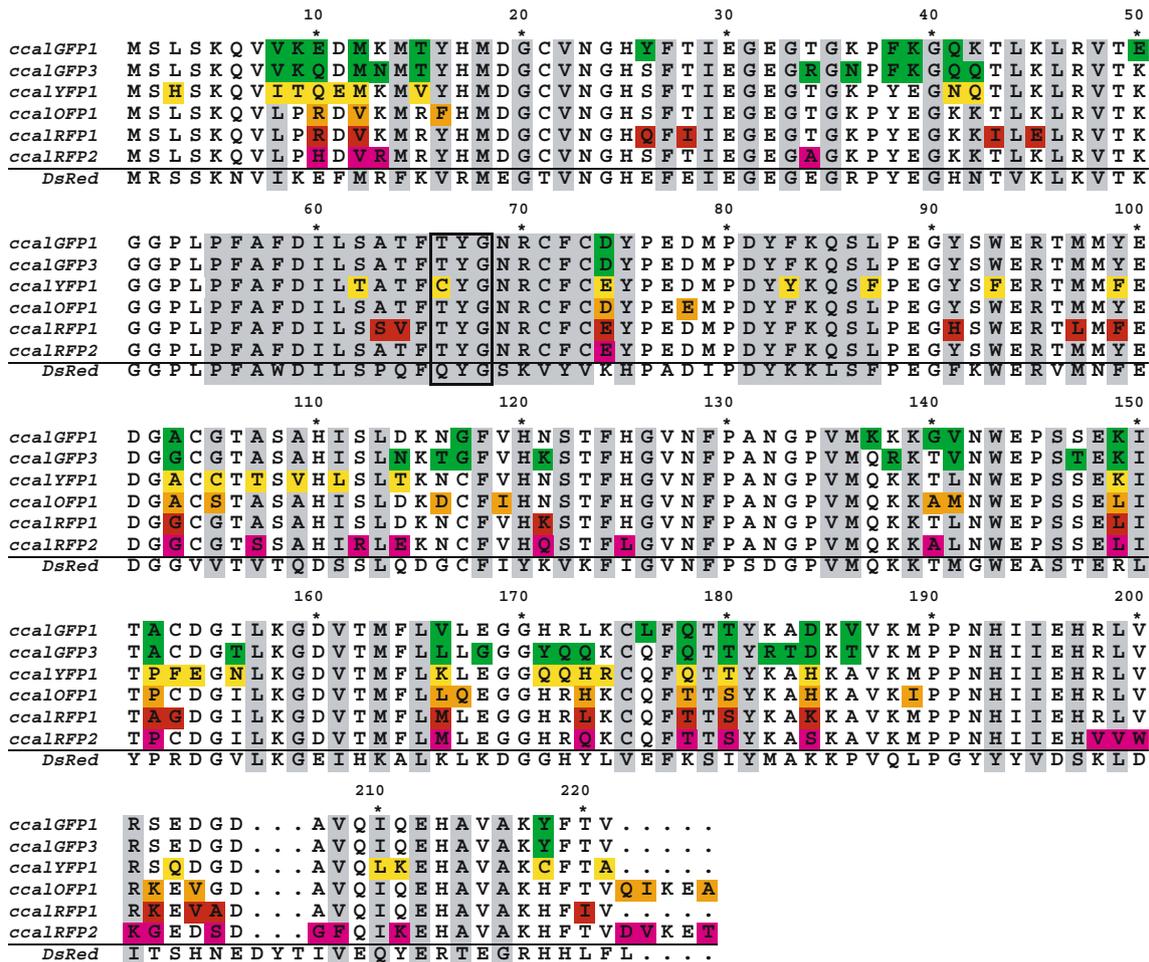


Fig. 2 Sequence alignment of the six *C. californica* fluorescent proteins. Variable residues (among ccalFPs) are colored by protein, with magenta for ccalRFP2, which has chromoprotein-like properties. The chromophore tripeptide sequence is boxed, and positions whose

sidechains lie on the inside of the β -can (in DsRed) are shaded grey. Numbering is according to the *Corynactis* proteins. The sequence of DsRed is shown below the line for comparison

none of these variables accounted for complete maturation to the red form of ccalRFP1. However, we found that ccalRFP1-expressing cells that were induced for extended periods (days) led to the production of the green-emitting species that could be purified in a stable form. In contrast, ccalRFP1-expressing cells that were induced for short periods of time (hours), and then rapidly purified and allowed to mature while exposed to air, formed the red-emitting chromophore. Other factors also seem to contribute to the differential maturation of ccalRFP1. For example, higher concentrations of protein also led to higher ratios of red-to-green emission in preparations where both forms were present. Although preliminary tests showed that oxygen levels could affect green-to-red maturation in ccalRFP1 (cf. Elowitz et al. 1997), these observations must be confirmed with additional experiments. Nevertheless, this general strategy allowed us to prepare fully mature green and red forms of ccalRFP1 (Fig. 3; Table 2); once matured, the green and red forms are stable indefinitely under constant conditions.

The green form of ccalRFP1 shows an absorbance maximum at 504 nm. Following excitation at 480 nm, the protein emits at 517 nm. In contrast, the red form of ccalRFP1 shows a broad absorbance maximum at ~568 nm at pH 8; red fluorescence at 598 nm is observed after excitation at 500 (and at longer wavelengths).

To characterize the green-to-red transition in the red form of ccalRFP1, we monitored fluorescence emission from rapidly prepared purified protein (Fig. 4). Initially, the protein shows strong green fluorescence at 517 nm. Over the course of ~16 h, green-emission diminishes with a concomitant increase in red fluorescence at 598 nm. The loss of green emission is likely due to efficient FRET

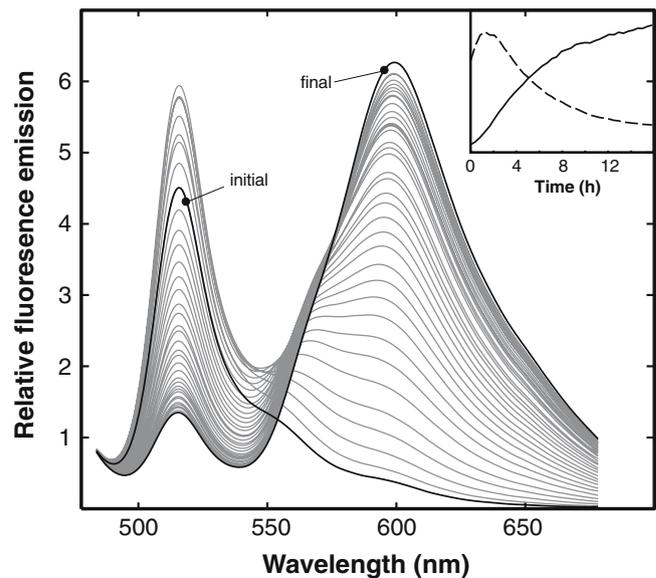


Fig. 4 Time-dependent maturation of the red-emitting chromophore in ccalRFP1. Fluorescent emission spectra of ccalRFP1, excited at 470 nm during the course of fluorescence development. The starting and ending spectra are drawn in black; intermediate spectra are in gray. (Inset) Time-course of green (dashed line; 515 nm) and red (solid line; 599 nm) fluorescence at each time point, taken at room temperature. Zero hours refers to the first measurement performed on freshly purified protein

between the 504 nm- and 568 nm-absorbing chromophore species in the fully mature ccalRFP1 dimer/tetramer.

Sequence and Phylogenetic Analysis

An amino acid alignment (Fig. 2; Table 3), shown relative to DsRed, shows regions of similarity among *Corynactis californica* fluorescent proteins. The ccalRFP2

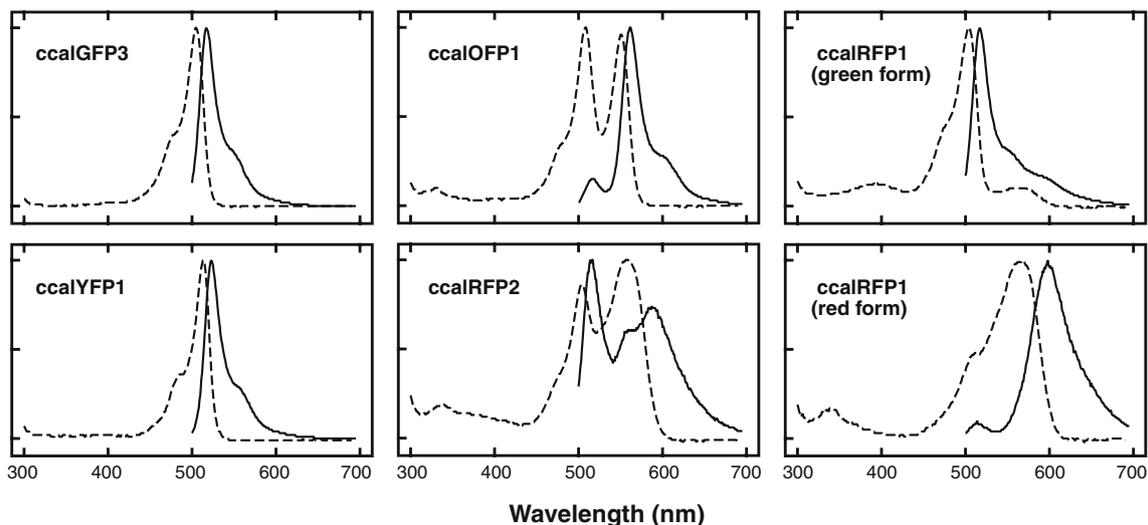


Fig. 3 Normalized absorbance (dashed line) and emission (solid line) spectra for each of the five purified *C. californica* fluorescent proteins. Spectra are shown for both forms (green and red) of ccalRFP1

Table 3 Fluorescent protein genes included in the phylogenetic analyses

Species Name	Protein Name (Common Name)	Genbank Accession Number	Excitation λ_{\max} (nm)	Emission λ_{\max} (nm)
<i>Agaricia fragilis</i>	afrag	AAK71331	U	U
<i>Astrangia lajollaensis</i>	alajGFP1	AAS18270	509	517
	alajGFP2	AAS18271	509	517
	alajGFP3	AAS18272	494	504
<i>Anemonia majano</i>	amajCyFP	AX824719	U	U
	amajGFP	AAF03371	458	486
<i>Anemonia sulcata</i>	asulCP	AAG41206	N/A	N/A
	asulGFP	AAN52735	U	U
<i>Cerianthus</i> sp.	ceriOFP	AAP55761	548	565
<i>Dendronephthya</i> sp.	dendRFP*	AAM10625	U	U
<i>Discosoma</i> sp. 1	discRFP (DsRed)	AAF03369	558	583
<i>Discosoma</i> sp. 2	dis2RFP	AAG16224	573	593
<i>Discosoma striata</i>	dstrCyFP	AAF03370	456	484
<i>Entacmaea quadricolor</i>	equaRFP	AAN05449	559	611
<i>Goniopora tenuidens</i>	gtenCP	AAL27542	N/A	N/A
<i>Lobophyllia hemprichii</i>	lhempRFP	AAV54099	571	581
<i>Montastraea cavernosa</i>	mcavCyFP	AAL17905	431	474; 497
	mcavGFP2	AAU04448	U	U
	mcavRFP	AAK71336	572	580
<i>Ptilosarcus</i> sp.	ptilGFP	AAG54097	500	508
<i>Ricordea florida</i>	rfloGFP1	AAK71338	508	518
	rfloGFP2	AAK71340	U	U
	rfloRFP	AAK71339	506; 566	517; 574
<i>Renilla muelleri</i>	rmueGFP	AAG54098	485	506
<i>Renilla reniformis</i>	rrenGFP	AAK54757	485	508
<i>Scolymia cubensis</i>	scubGFP1	AAK71333	497	506
	scubRFP1	AAU06843	U	576
<i>Trachyphyllia geoffroyi</i>	tgeoRFP (Kaede)	BAC20344	572	582
<i>Zoanthus</i> sp.	zoanGFP	AAF03372	U	506
	zoanRFP	AAL23574	552	576
	zoanYFP	AAF03373	U	538

U denotes that the information is unavailable. *Note: dendRFP was originally published as a green protein, dendGFP, by Labas et al. 2002

and ccalOFP1 are five amino acids longer than the other ccalFPs. The additional residues occur at the end of the sequence and not as insertions (Fig. 2), whereas there is one insertion with respect to DsRed. The chromophore of all ccalFPs was TYG, except for ccalYFP1, which had cysteine in place of the threonine.

The ccalFP genes possess 84% to 90% nucleotide and 75% to 89% amino-acid sequence identity to each other (Table 4). At the amino acid level, ccalGFP1 is most similar to ccalGFP3 (89% identical), ccalOFP1 is most similar to ccalRFP1 and ccalRFP2 (85%), whereas ccalYFP1 is most similar to ccalGFP1 (80%) and least related to ccalRFP2 (75%). FPs from *Anemonia majano*, the most similar FPs to the *C. californica* FPs, are 67% to 70% and 60% to 63% identical at the nucleotide and amino acid levels, respectively. Another FP in the same major clade as the *C. californica* FPs, zoanYFP, possesses 61% to 63%

nucleotide and 52% to 56% amino acid identity to the group. DsRed, the most commonly studied red FPs has 55% to 60% nucleotide and 43% to 49% amino acid identity to the ccalFPs. The most dissimilar fluorescent proteins among the Anthozoa are ceriOFP from a “primitive” ceriantharian tube anemone, and rrenGFP from an octocoral (32–35% AA identity). Although they were not included in the phylogenetic analyses presented in this article, FPs from copepods and hydrozoans (Shagin et al. 2004) are even more distantly related to the anthozoans presented herein. The *Corynactis* FPs are ~28% identical (at the amino-acid level) to the hydrozoan *Aequorea victoria* GFP, and ~26% identical to the copepod FPs.

Bayesian, maximum likelihood, and maximum parsimony analyses all reconstructed gene trees with strong support for the monophyly of ccal proteins, and for the grouping of the FPs with two other anthozoan genera: *Anemonia* and

Table 4 Pairwise amino acid identity of six *C. californica* fluorescent proteins

Fluorescent protein	Amino Acid Identity (%)					
	GFP1	GFP3	YFP1	OPF1	RFP1	RFP2
ccalGFP1	–	89	80	83	83	79
ccalGFP3		–	79	79	79	77
ccalYFP1			–	78	78	75
ccalOPF1				–	85	85
ccalRFP1					–	83
ccalRFP2						–

These proteins are more closely related to each other than to other common FPs, including *A. victoria* GFP (~27% identity), zoanYFP (~51% identity), ceriOPF (~38% identity), amajGFP (62%), and DsRed (~47% identity)

Astrangia (Fig. 5). In all of the consensus trees, ccalRFP1 and ccalOPF1 are grouped together, with strong support for ccalRFP2 as sister to them. CcalGFP1 and ccalGFP3 fall between the three long-wavelength proteins and ccalYFP1. Sister to the ccalFP group is a subgroup of proteins from *Anemonea majano* (amajCyFP and amajGFP) and *Astrangia lajollaensis* (alajGFP). The next closest neighbors of the ccalFPs include FPs from *Zoanthus* sp. (zoan2RFP, zoanYFP, zoanGFP).

Discussion

Properties of the *C. californica* Fluorescent Proteins

The six new *C. californica* fluorescent proteins described here provide an evolutionary snapshot of sequence and color diversity within a single organism. These proteins share ~75–89% amino-acid identity, including strict conservation of the chromophore tripeptide sequence (–TYG–) in all cases except for ccalYFP1 (–CYG–). However despite these similarities, they show dramatic differences in absorption, emission, brightness (the product of quantum yield and extinction coefficient) and maturation kinetics. These variations of biochemical and photophysical properties most likely result from sidechain alterations affecting the chromophore environment. A detailed understanding of these differences awaits high-resolution structural information.

The *C. californica* protein ccalYFP1 belongs to a small subset of naturally occurring yellow fluorescent proteins (>520 nm; Ward 2005). This group includes zoanYFP from *Zoanthus* (zoanYFP; Matz et al. 1999), and phiYFP from the hydromedusa *Phialidium* (phiYFP; Shagin et al. 2004). The unusual chromophore sequence of ccalYFP1 (–CYG–; Fig. 2) is found only in two green FPs from *Astrangia lajollaensis*, a temperate stony coral (Bessette

and Daugherty 2004). The key mutation conferring yellow emission to the commercially available GFP mutant YFP is T203Y, which shows coplanar π - π stacking with the chromophore resulting in a 16-nm red-shift in emission from 512 to 528 nm (Ormö et al. 1996; Wachter et al. 1998). Similar effects are observed when other aromatic amino acids, including tryptophan, phenylalanine and histidine, are substituted at this position. The corresponding residue in all of the *Corynactis* FPs (including ccalYFP1) is His197. This residue likely contributes to the red-shifted emission maximum relative to GFP that is seen with ccalGFP3, ccalYFP1, and the green form of ccalRFP1 (~517–523 nm) (Table 2; Fig. 3).

The *C. californica* protein ccalOPF1 joins the small group of naturally occurring orange-emitting fluorescent proteins (emission max \leq 580), which includes an OPF from the cnidarian tube-dwelling anemone, *Cerianthus* (Ip

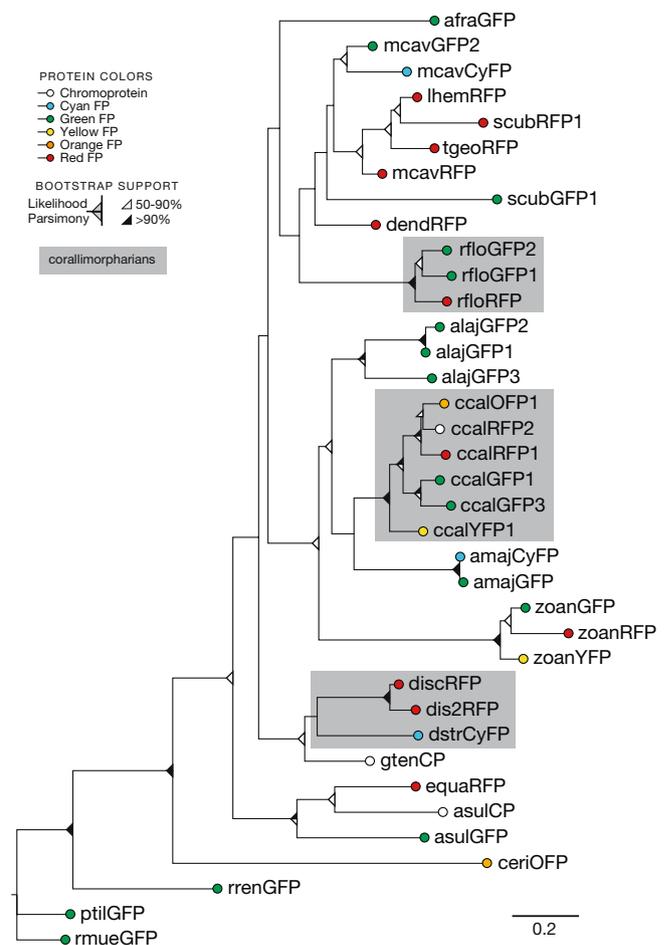


Fig. 5 Phylogenetic trees showing the relationship of *Corynactis californica* fluorescent proteins relative to each other and to other anthozoan FPs. Bootstrap support for each node is indicated by triangles above (maximum likelihood) or below (maximum parsimony) the line. Solid triangles above the line (>90% max. likelihood support) also all had Bayesian posterior probabilities of 1.0

et al. 2007), eqFP578 from the sea anemone *Entacmaea quadricolor* (Merzlyak et al. 2007), an OFP from the scleractinian *Lobophyllia hemprichii* (Oswald et al. 2007), and the Kusabira-orange “KO” FP from the stony coral *Fungia concinna* (Karasawa et al. 2004). (The latter incomplete protein was modified by the addition of 7 residues to the N-terminus). The structural basis of orange-emission in naturally occurring OFPs is not yet understood in detail. In mOrange (an engineered, monomeric variant of DsRed; Shaner et al. 2004) the sidechain oxygen of Thr66 forms a covalent bond with the carbonyl carbon of Phe65 to form an additional five-membered ring in the chromophore (Shu et al. 2006). CcalOFP1 contains the same chromophore sequence as mOrange (–TYG–), but whether orange-emission in ccalOFP1 requires a similar covalent modification of the chromophore awaits further analysis.

The most unique of the *C. californica* FPs is ccalRFP1. At 598 nm, its fluorescence is one of the longest-wavelength emissions known from a natural protein, with the longest being eqFP611 (Wiedenmann et al. 2002). Under the appropriate conditions, ccalRFP1 exhibits a “fluorescent timer” phenotype, converting from a green- to a red-emitting protein over a period of ~16 h at room temperature (Figs. 3 and 4). Maturation does not seem to be light-dependent. Preliminary analysis of absorbance during the progression from green-to-red (data not shown) suggests that ccalRFP1 maturation proceeds via a branched pathway similar to that proposed for DsRed (Verkhusha and Lukyanov 2004). In this model, a transient, protonated GFP-like intermediate (absorbing maximally at ~408 nm) leads to a “dead-end” green-emitting chromophore or to a red-emitting chromophore. The bright red fluorescence observed in mature ccalRFP1 is due to efficient FRET between the 504 nm- and 568 nm-absorbing chromophore species in the dimer/tetramer.

Time-dependent fluorescence changes are useful for tracking changes in gene expression in vivo, as described previously for the engineered DsRed variant, E5 (Terskikh et al. 2000). For other applications, rapid and complete maturation to a red-emitting species is desired. Green-to-red maturation of ccalRFP1 is faster than that observed with the original red fluorescent protein, DsRed, which requires >48 h to reach 90% maximal fluorescence at room temperature (Baird et al. 2000), but it is markedly slower than engineered DsRed variants that mature to a stable end point in less than 2 h (Bevis and Glick 2002, Campbell et al. 2002).

The discovery of a set of closely related but phenotypically unique fluorescent proteins from *C. californica* provides a unique opportunity to study the molecular basis of color diversity in the GFP family. In addition, the *Corynactis* FPs provide a new starting point for protein

engineering efforts geared toward optimization for specific imaging applications.

Multi-Locus Nature of Pigmentation

The data presented show the same suite of at least three to five homologous FP genes (and possibly six or more) is expressed in seven color morphs of *Corynactis californica* collected at various depths from Monterey Bay, CA (Table 1). This evidence indicates that pigmentation is a multi-locus trait in this species, since the number of unique alleles per locus should not exceed two in a diploid organism. The nucleotide differences among the six FP genes (10–16%) also are greater than what would be expected for different alleles of the same locus. It has been shown in other anthozoans that FP genes can be represented at several loci (Kelmanson and Matz 2003).

The variation in pigment colors and patterns among morphs presumably results from varying the expression level of each protein in specific tissues at some stage in development. This points to independent control of gene expression by tissue type and also supports the multi-locus nature of pigmentation. This is clear in the Pink2, Orange1, and Red morphs, which all have major emission peaks around 600 nm in all tissues (Table 1), and appear red under blue excitation (Fig. 1h,j,k), but are expressing yellow and orange proteins together with red proteins (Table 1). The yellow and orange proteins are presumably expressed at much lower levels than the red proteins in these morphs. Alternatively, the yellow and orange proteins may not have been detected in vivo because they are participating in co-localized energy transfer (such as FRET), with their emission energy channeled into added excitation energy for the red proteins (Gilmore et al. 2003). It is not always the case that the presence of red proteins will determine the final perceived emission; the Orange2 morph has a dominant emission peak of 562 nm in all tissues (Table 1) and appears orange under blue light (Fig. 1i), even though the expression of red proteins (ccalRFP1 and ccalRFP2) was detected along with ccalOFP1 (Table 1).

Additional evidence for the genetic basis of diverse coloration derives from Pattern B morphs (Fig. 1e–g): All express the same suite of FP genes (Table 1), though spectral differences were detected within tissues among the three morphs (Table 1). The spectral variation is due to tissue-specific differences in expression levels of specific FPs, resulting in different patterns (Fig. 1l–n). These results confirm that the evolutionary basis of fluorescence in *C. californica* is not simple single locus mechanism. The data indicate that certain morphs contain all but one or two of the six genes in their genomes, so underlying differences in inheritance (polymorphisms) may exist among certain morphs, especially between morphs exhibiting either

Pattern A or B. Alternatively, all morphs could contain all six FP genes in their genomes, but expression was not detected with the methods used. Expression levels could be mediated at some point in the life cycle by certain environmental parameters (phenotypic plasticity). Further studies need to be performed to determine the influence of genes versus environment on the expression levels of fluorescent proteins in this species.

Sequence and Phylogenetic Analyses

Whereas green proteins only require two reactions to complete their chromophore maturation, red fluorescent proteins require three consecutive autocatalytic reactions, resulting in an extended, more complex chromophore structure (Gross et al. 2000; Yarbrough et al. 2001). The evolution of red emission from green has been seen multiple times in the GFP family, and may represent convergent evolution of complexity (Shagin et al. 2004; Ugalde et al. 2004), in the sense that the chemical process for attaining red emission is more involved. This also seems to be true for proteins from *C. californica*.

The consensus of the trees reconstructed by ML, Bayesian, and MP analyses suggests that the red and orange proteins in *C. californica* evolved together and are more derived with respect to GFPs of sister taxa than is the yellow protein (Fig. 5). This may indicate that residues that contribute to orange or red emission may have evolved together from a green ancestor, as found in other systems.

As expected, the six genes from *C. californica* cluster together. However, the sister protein to the *Corynactis* FPs is the FP from the actiniarian *Anemonia majano* (amajGFP), with GFPs from scleractinian *Astrangia* and the zoanthid *Zoanthus* joining the overall clade. Other corallimorpharian FPs, represented by *Discosoma* and *Ricordea*, do not group with the ccalFPs, and are positioned independently in other parts of the tree (Fig. 5, grey highlighted areas). This indicates multiple diversification events of fluorescence even within the corallimorpharian groups or possibly a polyphyletic corallimorpharia, rather than multiple occurrences of lateral gene transfer or some other mechanism. Thus as natural selection acts on color or chromophore phenotype, color evolution is not tightly restricted by shared ancestry. More molecular phylogenetic studies of the basic relationships between corallimorpharian, scleractinian, and actiniarian species are needed before further conclusions regarding the evolution of FP genes in these groups can be made (Matz et al. 2002). As has been done with the great star coral *Montastrea cavernosa* (Kelmanson and Matz 2003), it also will be interesting to examine the genomic loci of the *Corynactis californica* FPs to see how the protein expression patterns are regulated and determined.

Biological Significance

The function of fluorescent proteins in marine organisms remains an open question. Symbiotic organisms are thought to use green FPs to regulate the amount and wavelength of sunlight reaching their algal endosymbionts, either through photoprotection or through photoenhancement. In photoprotection, shallow-dwelling organisms use fluorescent pigments to redirect harmful excess sunlight away from symbionts (Kawaguti 1944; Salih et al. 2000; Gilmore et al. 2003). In photoenhancement, light-limited organisms use FPs or other pigments to absorb short wavelengths of light, transform them to longer wavelengths more suitable for photosynthesis, and emit them to symbionts (Kawaguti 1969; Schlichter et al. 1994).

Light-regulation proposals, however, fail to account for the presence of GFP-type pigments in non-bioluminescent, nonsymbiotic organisms, for the wide range of fluorescent colors found in nature, or for the multicolored patterns seen in species such as *Corynactis californica* (Shagin et al. 2004; Wiedenmann et al. 2004). In *C. californica*, which does not harbor endosymbiotic algae, it is unlikely that FPs function in photoenhancement or photoprotection. Although the *Corynactis californica* FPs might serve to protect polyps from extreme variations in light, a distributional study of a closely related species (*Corynactis viridis*) showed that polyps retracted when exposed to increased levels of direct sunlight, and some showed rhythmic activity (expanding by night and retracting by day) (Muntz et al. 1972). Given that response mechanisms to light levels are already present in these organisms, it seems that the function of fluorescent pigmentation in *Corynactis californica* is unrelated to the regulation of light levels. Thus, FPs likely have evolved multiple functions in the marine environment.

Fluorescence may provide a visual contrast that functions as a signal in interspecies relationships (Ward 2002). Conspicuous coloration as a form of communication has been investigated in terrestrial and aquatic environments (Cott 1957). Colors may signal to predators or competitors that an organism is toxic or dangerous. Although *C. californica* lacks any specialized visual system, it is possible that, with excitation from blue downwelling sunlight, its fluorescent pigments act as a signal for interspecies communication. Known predators of *C. californica* (*Calliostoma annulatum*, *Latiaxis oldroydi*, *Dermasterias imbricata*, and *Pisaster giganteus*) have limited visual ability and principally rely on chemical cues to search for prey, therefore, they likely would not respond to fluorescent signals.

Prey attraction via fluorescent “lures” was recently suggested for a species of deep-sea siphonophore (Haddock et al. 2005), and a similar a function could perhaps be utilized by *Corynactis californica*. Potential prey, including

crustaceans and fish, typically possess the visual systems and pigments required to detect fluorescence, and fluorescent proteins could provide appropriate visual signals (Marshall 2000; Losey et al. 2003; Marshall et al. 2003a; b; Matz et al. 2006). Additional studies will be necessary to explore correlations between the visual pigments of target species and fluorescent signals from organisms, such as *Corynactis californica*.

The recent discovery of FP genes in members of the Ceriantharia including a green protein from *Cerianthus membranaceus* (Wiedenmann et al. 2004) and an orange protein from *Cerianthus* sp. (Ip et al. 2004; Ip et al. 2007) is interesting because this group is nonsymbiotic and believed to be basal within the Zoantharia (France et al. 1996; Bertson et al. 1999). If FP genes derive from an ancestor to this nonsymbiotic group, it is possible that FPs originally evolved for reasons other than light regulation, and that organisms with symbionts evolved this function later. This information, together with data presented in the present study, provides strong evidence for multiple functions of the wide range of FPs within and beyond the Anthozoa.

This study demonstrates that the “neon glow” of *Corynactis californica*, alluded to by Wicksten (1989) and others (Limbaugh and North 1956; West 1979; Schmieder 1991), is the result of a series of fluorescent proteins expressed in *Corynactis californica* color morphs. The demonstration that a nonsymbiotic anthozoan has evolved multiple FPs of varying emission color, and that intraspecific variation in pigmentation is determined by the tissue-specific expression of these proteins, suggests that organisms may utilize FPs for reasons beyond light regulation, such as interspecies communication. Our data show that fluorescent pigmentation is a multi-locus trait, with three to five loci expressed in each morph. If fluorescent pigmentation is a plastic character, the expression of individual FPs should vary with specific environments, and future studies may uncover factors influencing patterns and levels of expression in *C. californica*. The multi-locus nature of pigmentation in *C. californica* and in related species with fluorescent proteins may allow for broad distribution and acclimatization to shifting external conditions and species interactions.

Conclusions

We have cloned a set of six closely related GFP-like proteins from seven *C. californica* color morphs. These genes encode proteins that fluoresce across the green-to-red visible spectrum, including one that exhibits a “fluorescent timer” phenotype in vitro. Sequence and phylogenetic analysis indicate that these genes arose together in the genus, but if current scleractinian classification is correct, corallimorpharian FPs diverged in an ancestor to the

Anthozoa before speciation events separating anthozoan subclasses.

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