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Molecular evidence for color discrimination in the Atlantic sand fiddler crab, *Uca pugilator*

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SUMMARY

Fiddler crabs are intertidal brachyuran crabs that belong to the genus *Uca*. Approximately 97 different species have been identified, and several of these live sympatrically. Many have species-specific body color patterns that may act as signals for intra- and interspecific communication. To understand the behavioral and ecological role of this coloration we must know whether fiddler crabs have the physiological capacity to perceive color cues. Using a molecular approach, we identified the opsin-encoding genes and determined their expression patterns across the eye of the sand fiddler crab, *Uca pugilator*. We identified three different opsin-encoding genes (*UpRh1*, *UpRh2* and *UpRh3*). *UpRh1* and *UpRh2* are highly related and have similarities in their amino acid sequences to other arthropod long- and medium-wavelength-sensitive opsins, whereas *UpRh3* is similar to other arthropod UV-sensitive opsins. All three opsins are expressed in each ommatidium, in an opsin-specific pattern. *UpRh3* is present only in the R8 photoreceptor cell, whereas *UpRh1* and *UpRh2* are present in the R1-7 cells, with *UpRh1* expression restricted to five cells and *UpRh2* expression present in three cells. Thus, one photoreceptor in every ommatidium expresses both *UpRh1* and *UpRh2*, providing another example of sensory receptor coexpression. These results show that *U. pugilator* has the basic molecular machinery for color perception, perhaps even trichromatic vision.

Key words: color vision, photoreceptor, visual pigment, rhodopsin, *Uca pugilator*, fiddler crab.

INTRODUCTION

Animal coloration has long been implicated in intraspecific signaling (Darwin, 1871), particularly in the context of sexual selection and mate choice. Not surprisingly, the ability to visually distinguish colors has been found to be a major sensory cue for these behavioral and ecological functions in many species [e.g. primates (Fernandez and Morris, 2007), spiders (Lim et al., 2008), cichlids (Seehausen et al., 2008), crabs (Baldwin and Johnsen, 2009), stomatopods (Cheroske et al., 2009) and frogs (Sztatecsny et al., 2010)]. Research using fiddler crabs (genus *Uca*) has provided several insights into the mechanisms of intraspecific visual signaling and communication, but this work has generally focused on form and motion cues or, if it involved the manipulation of color, could not rule out achromatic contrast as a confounding visual cue (for reviews, see Crane, 1975; Salmon and Hyatt, 1983; Christy and Salmon, 1984; Salmon, 1984; Salmon and Zucker, 1988; Christy and Salmon, 1991; Backwell and Passmore, 1996; Koga et al., 1998; Backwell et al., 1999; Backwell et al., 2000; Christy et al., 2001; Murai and Backwell, 2005; Christy and Backwell, 2006; How et al., 2008). To summarize a few salient findings, the males' claw-waving displays attract the attention of females, and because the waves' spatial and dynamic aspects are species specific, it probably also communicates species identity (Aspey, 1971; How et al., 2009). The conspicuousness of this display is aided by the enhanced visual contrast of the carapace and major cheliped against a mud background, provided by their particular spectral reflectance, which can even change to maintain high contrast after a change in background (Zeil and Zanker, 1997; Zeil and Hofmann, 2001; Zeil and Hemmi, 2006). When female *U. mjoebergi* and *U. signata* were given a choice between hetero- and conspecific males with painted chelae they preferred whichever one was painted to resemble a conspecific male (Detto et al., 2006).

Only a few behavioral experiments have been specifically designed to test for color vision in fiddler crabs, and these have been fairly successful. Female *U. pugilator* showed positive phototaxis towards monochromatic blue and red–orange light, but not towards white light (Hyatt, 1974). Recently, when *U. mjoebergi* females were subjected to a choice between males having a yellow claw and males whose claws had been painted one of several shades of grey, they preferred the yellow claw, suggesting that mere luminosity does not mediate the discrimination (Detto, 2007). Thus, for *U. mjoebergi*, coloration does not merely increase luminance contrast, but is a real color signal in both visible and ultraviolet wavelengths (Detto and Backwell, 2009).

Despite the evidence for color vision from behavioral studies, attempts to demonstrate color vision physiologically have yielded mixed results. In an early attempt, glass electrode electroretinograms (ERG) on *U. pugilator*, *U. pugnax* and *U. minax* produced broad spectral sensitivity curves with maxima between 450 and 500 nm, predicting at least two pigments with one sensitive in blue–violet and the other in red–orange (Hyatt, 1974). This contradicted results published in the same year from cotton wick ERGs from *U. pugilator* and *U. pugnax*, which showed an absorption curve with only a single peak at ~508 nm (Scott and Mote, 1974). Both studies also included selective adaptation of photopigments with monochromatic light, and both studies reported results that supported their opposing conclusions from the ERG amplitudes, adding to the confusion over whether these species have one or more visual pigments. Much later, action potentials recorded from the optic nerve of *U. thayeri* showed broad spectral response curves that were best fit by two visual

Table 1. Primers used to sequence *U. pugilator* opsins

Primer name	Primer description	Sequence (5′→3′)	Melting point (°C)
F5	Degenerate (LW)	GCG AAT TCC GSG ANC ARG CMA ARA RRW TG	59.6
R8	Degenerate (LW)	CGG GAT CCD AYR SMR TAN ACR AWN GG	51.9
UpDF1	Degenerate (SW)	CAT GTT CAT CAT CAA CCT GGC VAW RAA CC	58.5
Hybrid dT	Specific (3′RACE)	GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT	57.4
Adapter R	Specific (3′RACE)	GAC TCG AGT CGA CAT CG	52.2
UpRh1 R1	Specific (5′RACE)	ACA TTG GAT TCA CGG GAG CGT AGT	60.3
UpRh2 R1	Specific (5′RACE)	TTA GCT TCA AAC GGC TTG GCG ATG	60.2
UpRh3 R1	Specific (5′RACE)	ATG AGA ACA CGA AGA TGG CAC CGA	60.4

pigments having peak wavelength of spectral sensitivities (λ_{max}) at 430 nm and 510–530 nm (Horch et al., 2002). Finally, recent microspectrophotometric measurements from *U. tangeri*, *U. vomeris*, *U. pugnax* and *U. pugilator* showed only one visual pigment present in retinula cells 1–7 (R1–R7 out of a total of eight) with λ_{max} ranging from 500–540 nm depending on the species (Jordao et al., 2007). The possibility of a different visual pigment in the eighth retinula cell (R8) was not ruled out since the small size of these cells precluded any measurements.

Thus, although behavioral studies tend to indicate color perception, these have only occasionally been corroborated by physiological evidence. This disparity may exist because electrophysiological and spectrophotometric measurements tend to be of poor quality in *Uca*. The poor quality is most likely due to the enormous amount of screening pigments, which serve several functions, including restricting stray light, meta-rhodopsin conversion and light adaptation (Eguchi and Waterman, 1967; Ribi, 1978; Stavenga, 1989). Such studies therefore often produce results that only weakly support one side or the other.

The minimum requirement for color vision is the expression of at least two active opsins of different spectral absorbance in at least two different members of the eight photoreceptor cells comprising an ommatidium (also called retinula cells R1–R8). The present study aims to identify the physiological substrate for color vision in fiddler crabs, if it exists. We avoid the problems associated with the screening pigments by using molecular biological techniques. We identified three distinct opsin transcripts (*UpRh1*, *UpRh2* and *UpRh3*) in the retinas of both male and female *U. pugilator* and found by *in situ* hybridization that all three opsins are expressed in all ommatidia. An ostensibly UV-sensitive opsin, *UpRh3*, was found in R8 and, of the remaining seven retinula cells, five were found to have *UpRh1* and three to have *UpRh2*, meaning there is coexpression of two of the opsins in a single retinula cell in each ommatidium.

MATERIALS AND METHODS

Animals

Uca pugilator (Bosc 1802) sand fiddler crabs were collected from Beaufort, NC, USA and maintained at room temperature in the laboratory (Cincinnati, OH, USA) in a tank containing soil from their native habitat. The enclosure was filled and emptied at six hour intervals with 20 parts per thousand salinity water (Instant Ocean, Spectrum Brands Inc., Alpharetta, GA, USA), thereby mimicking a 12 h circatidal cycle. Crabs were placed on a 12 h:12 h L:D cycle and fed pulverized fish food (Tetramin, Spectrum Brands Inc.).

RNA isolation, cDNA synthesis and PCR amplification

Total RNA was extracted from the eyes of five *U. pugilator* male fiddler crabs using TRIzol reagent according to the manufacturer's protocol (Gibco BRL, Gaithersburg, MD, USA). Single-stranded complementary DNA (cDNA) was synthesized from ~1 μg of total RNA using oligo(dT) primers provided with the Affinityscript QPCR cDNA synthesis kit (Stratagene, La Jolla, CA, USA). Synthesized cDNA was used in PCR reactions with degenerate primers F5 and R8 designed based on the conserved regions found in all arthropod opsins (Hariyama et al., 1993) (Table 1). Owing to the presence of excess amount of polysaccharides in the crustacean eye tissue, Plant RNA Isolation Aid (Applied Biosystems, Foster City, CA, USA) was used in every PCR reaction (1:10 dilution per reaction) to prevent PCR inhibition. PCR amplifications were typically performed in 25 μl reaction volumes consisting of: 2.5 μl PCR 10× buffer, 2.5 μl 25 mmol l^{-1} $MgCl_2$, 4 μl 10 mmol l^{-1} dNTPs, 1 μl 100 μmol l^{-1} forward primer, 1 μl 100 μmol l^{-1} reverse primer, 0.5 μl *Taq* DNA polymerase (Takara Bio Inc., Madison, WI, USA), 1.5 μl cDNA, 2.5 μl Plant RNA Isolation Aid and 9.5 μl H_2O. PCR conditions were as follows: 5 min of initial denaturation; 30 cycles of 1 min at 94°C, 1 min at 48–55°C (depending on the Tm of the primer pair), and 1 min at 72°C; and a final extension of 5 min at 72°C. The PCR products were separated on a 1% agarose gel, fragments of appropriate length were gel extracted (QIAquick Gel Extraction Kit; QIAGEN, Valencia, CA, USA), and these were cloned into pGEM-T Easy (Promega, Madison, WI, USA). Samples were bi-directionally sequenced with vector primers SP6 and T7. These partial opsin sequences were then used to design additional degenerate and opsin specific primers as listed in Table 1 for 5′ and 3′ RACE reactions.

3′ and 5′ RACE reaction

To obtain full-length opsin sequences, the 3′ and 5′ ends of the opsin genes were amplified using RACE. For 3′ RACE, cDNA was synthesized from ~1 μg of the total RNA as described above using the adapted oligo(dT) primer, Hybrid dT (Table 1). Adapter R primers (reverse complementary to the 5′ end of the Hybrid dT) were used as reverse primers in conjunction with opsin-specific forward primer. 5′ RACE reactions were performed from ~10 μg of total RNA according to the manufacturer's protocol with the Firstchoice RLM-RACE kit (Applied Biosystems) using opsin-specific reverse primers (*UpRh1* R1, *UpRh2* R1 and *UpRh3* R1) and kit-provided forward primers.

Phylogenetic analysis

Full-length *U. pugilator* opsin cDNA sequences were translated 'in silico' and aligned using CLUSTALW [MEGA 4.0 software (Tamura et al., 2007)] with other arthropod opsin protein sequences obtained from GenBank (species and accession numbers listed in Table 2). A neighbor-joining algorithm (Saitou and Nei, 1987) with Poisson correction (Zuckercandl and Pauling, 1965) was used to construct an opsin tree, and bootstrapped by replicating 1000 times. Pairwise deletion algorithm was also used to eliminate any alignment gaps present in the sequence.

Table 2. Arthropod opsin protein sequences used in phylogenetic analysis

Opsin class	Species	Taxon	GenBank accession no.	References
LW	*Apis mellifera*	Insecta	U26026	Townson et al., 1998
	Drosophila melanogaster Rh1	Insecta	K02315	Feiler et al., 1992
	D. melanogaster Rh2	Insecta	M12896	Feiler et al., 1992
	D. melanogaster Rh6	Insecta	Z86118	Salcedo et al., 1999
	Limulus polyphemus lateral eye	Chelicerata	L03781	Smith et al., 1993
	L. polyphemus ocelli	Chelicerata	L03782	Smith et al., 1993
	Papilio xuthus Rh1	Insecta	AB007423	Arikawa et al., 1987
	P. xuthus Rh2	Insecta	AB007424	Arikawa et al., 1987
	P. xuthus Rh3	Insecta	AB007425	Arikawa et al., 1987
	Pieris rapae	Insecta	AB177984	Ichikawa and Tateda, 1982
	Schistocera gregaria	Insecta	CAA56377	Towner et al., 1997
	Tribolium castaneum	Insecta	XM_968054	Jackowska et al., 2007
	Thermonectus marmaratus LW	Insecta	EU921225	Maksimovic et al., 2009
	Vanessa cardui	Insecta	AF385333	Briscoe et al., 2003
MW	*Calliphora vicina* Rh1	Insecta	M58334	Paul et al., 1986
	D. melanogaster Rh1	Insecta	K02315	Feiler et al., 1992
	D. melanogaster Rh2	Insecta	M12896	Feiler et al., 1992
	Hemigrapsus sanguineus Rh1	Crustacea	D50583	Sakamoto et al., 1996
	H. sanguineus Rh2	Crustacea	D50584	Sakamoto et al., 1996
	Portunus pelagicus	Crustacea	ABM74400	Kuballa et al., 2007
	Triops granarius opsin1	Crustacea	BAG80976	Kashiyama et al., 2009
	T. granarius opsin2	Crustacea	BAG80977	Kashiyama et al., 2009
	T. granarius opsin4	Crustacea	BAG80979	Kashiyama et al., 2009
	T. granarius opsin5	Crustacea	BAG80980	Kashiyama et al., 2009
	Triops longicaudatus opsin1	Crustacea	BAG80981	Kashiyama et al., 2009
	T. longicaudatus opsin2	Crustacea	BAG80982	Kashiyama et al., 2009
	Uca vomeris 1	Crustacea	GQ228846	–
	U. vomeris 2	Crustacea	GQ228847	–
SW	*A. mellifera*	Insecta	AF004169	Townson et al., 1998
	Acyrthosiphon pisum	Insecta	XP_001951588	Pruitt et al., 2007
	Bicyclus anynana	Insecta	AAL91507	Vanhoutte et al., 2002
	Bombus impatiens	Insecta	AAV67326	Spaethe and Briscoe, 2005
	Branchinella kugenumaensis opsin 1	Crustacea	BAG80984	Kashiyama et al., 2009
	B. kugenumaensis opsin 2	Crustacea	BAG80985	Kashiyama et al., 2009
	B. kugenumaensis opsin 3	Crustacea	BAG80986	Kashiyama et al., 2009
	Camponotus abdominalis	Insecta	AF042788	Smith et al., 1997
	Cataglyphis bombycinus	Insecta	AF042787	Smith et al., 1997
	D. melanogaster Rh3	Insecta	M17718	Feiler et al., 1992
	D. melanogaster Rh4	Insecta	M17730	Feiler et al., 1992
	D. melanogaster Rh5	Insecta	U67905	Salcedo et al., 1999
	Heliconius erato	Insecta	AAY16529	Pohl et al., 2009
	Manduca sexta	Insecta	L78081	White et al., 1983
	P. glaucus Rh5	Insecta	AF077191	Briscoe et al., 2000
	P. xuthus Rh5	Insecta	AB028218	Arikawa et al., 1987
	S. gregaria	Insecta	CAA56378	Towner et al., 1997
	T. castaneum	Insecta	XM_965251	Jackowska et al., 2007
	T. granarius opsin3	Crustacea	BAG80978	Kashiyama et al., 2009
	T. longicaudatus opsin3	Crustacea	BAG80983	Kashiyama et al., 2009
	T. marmaratus UV I	Insecta	EU921226	Maksimovic et al., 2009
	T. marmaratus UVII	Insecta	EU921227	Maksimovic et al., 2009
	V. cardui	Insecta	AF414074	Briscoe et al., 2003

The λ_{max} values used to classify the opsins were determined using electrophysiological measurements or predicted from molecular analysis.

Probe synthesis for *in situ* hybridizations

Riboprobes were generated from ~200 bp present at unique 3′ untranslated regions (3′UTR) or 5′ ends of the coding region (Fig. 1). Appropriate fragments were PCR amplified, cloned into pGEM T-Easy (Promega, Madison, WI, USA), and digoxigenin- and fluorescein-labeled antisense and sense (control) riboprobes were synthesized using the SP6/T7 transcription kit (Roche Applied Science, Indianapolis, IN, USA). After riboprobe synthesis, the template DNA was degraded using 2 µl of DNase, provided in the kit, for 15 min at 37°C. Synthesized probes were stored in hybridization buffer [0.3 mmol l^{-1} NaCl, 2.5 mmol l^{-1} EDTA, 20 mmol l^{-1} Tris-HCl (pH 8.0), 50% formamide, 10% dextran sulfate, 100 mg ml^{-1} yeast tRNA and 1× Denhardt's medium (Sakamoto et al., 1996)] at –80°C until further use.

Single *in situ* hybridization

Fiddler crabs (*U. pugilator*) were immobilized on ice and their eyes were dissected directly into RNA Later Solution (Applied Biosystems, Foster City, CA, USA). The eyes were then fixed with 4% paraformaldehyde in 1× phosphate-buffered saline (PBS; pH 7.4) at room temperature (RT) for 2 h. After fixation, the tissue was washed twice in 1× PBS for 15 min and dehydrated through an ethanol series. After two 1 h washes in xylene, the tissue was embedded in paraffin wax (Thermo Scientific, Wilmington, DE,

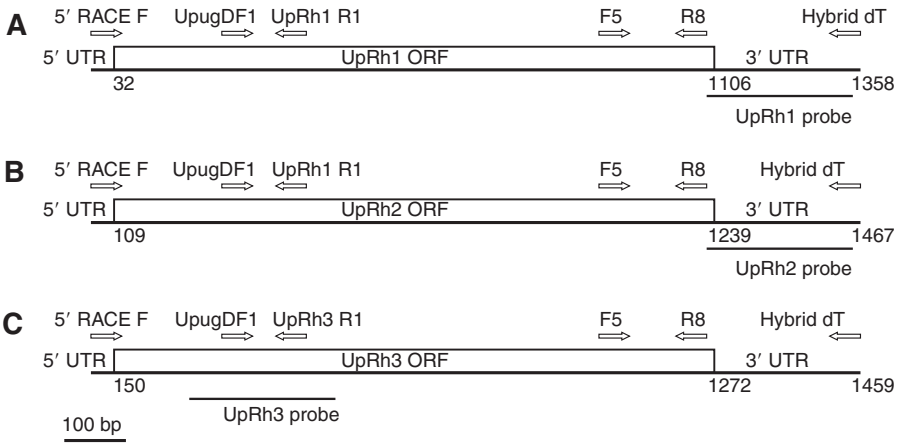

Fig. 1. Schematic of three opsin cDNA sequences identified from *U. pugilator* retina. (A) *UpRh1* cDNA, (B) *UpRh2* cDNA and (C) *UpRh3* cDNA. 5′ and 3′ untranslated regions (UTR) and the open reading frame (ORF) are indicated in the figure. The total length of the sequence and positions of the initiation and stop codons are also indicated. The location of the riboprobes synthesized for the *in situ* hybridizations are also shown with a line. Primers used to amplify the cDNA fragments are also shown.

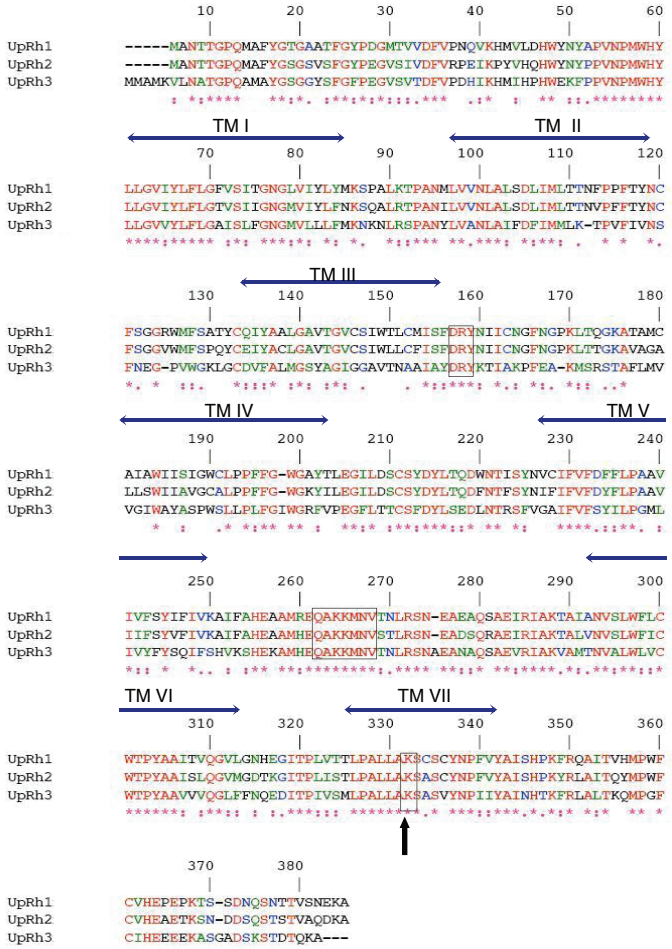

Fig. 2. Amino acid alignment of translated cDNA opsin sequences identified from *U. pugilator* using CLUSTALW. Predicted transmembrane domains of all three opsins are indicated by double-headed arrows. The conserved lysine involved in the Schiff's base linkage with the chromophore molecule is denoted with an arrow. Other possible conserved functional amino acids are boxed and the identical amino acids are indicated in red (and * below), strongly similar amino acids in green (:) and the weakly similar amino acids in blue (.).

USA). The embedded tissue was frozen at –20°C overnight, and ~10 μm paraffin sections were cut using a microtome (Leitz Wetzlar, Wetzlar, Hesse, Germany). Sections were then washed once in 1× PBS for 10 min, rehydrated in a graded ethanol series to remove the paraffin wax, and hybridized with 0.5 μg ml^{-1} of digoxigenin-labeled probe at 55–60°C for 18 h in hydridization buffer. After hybridization, the slides were washed at 55–60°C with 5× SSC, followed by sequential 30-min washes of 2×, 1× and 0.2× SSC buffer. Sections were then blocked with 10% normal sheep serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in 1× PBX for 1 h at RT, incubated at 4°C overnight with a 1:250 dilution of sheep anti-digoxigenin–AP (Roche Applied Sciences, Indianapolis, IN, USA) in blocking solution, and then washed three times in 1× PBX at RT for 20 min. Alkaline phosphate (AP) staining was developed using either the 1-STEP NBT/BCIP plus suppressor (Thermo Scientific, Wilmington, DE, USA) or with Fast Red tablets (Roche Applied Sciences, Indianapolis, IN, USA) according to manufacturer's instructions. After staining, the sections were washed twice for 10 min in 1× PBX at RT, rinsed with distilled H_2O and mounted using Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Images were digitally captured using an Olympus BX51 microscope equipped with an Olympus 60806 camera (Olympus America Inc., Center Valley, PA, USA) and processed with the Adobe Photoshop image processing software (Adobe Systems Inc., San Jose, CA, USA). Fluorescent images were captured with a Texas Red filter channel.

Double *in situ* hybridization

Double *in situ* hybridizations were performed as described above with minor modifications. Two different 0.5 μg ml^{-1} riboprobes labeled with digoxigenin and fluorescein, respectively, were used simultaneously in the hybridization buffer. Sections were blocked in a mixture of 10% normal sheep serum and 10% normal goat serum (Jackson ImmunoResearch) and tissue sections were simultaneously incubated at 4°C for 18 h with both sheep anti-digoxigenin–AP (1:250) and goat anti-fluorescein–POD (1:100; Roche Applied Sciences, Indianapolis, IN, USA) diluted in blocking solution. A fluorescein-labeled probe was detected using a DAB substrate kit (Vector Laboratories, Burlingame, CA, USA) according to manufacturer's instructions. Alkaline phosphatase activity was

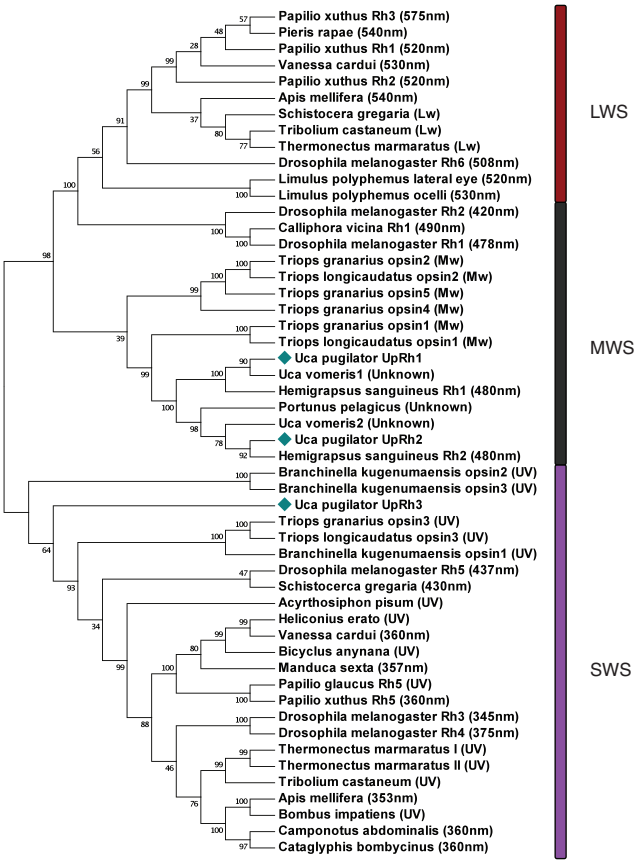

Fig. 3. Phylogenetic relationships of *U. pugilator* opsins with other known arthropod opsins. The *U. pugilator* opsins are highlighted with a colored diamond in the node. Numbers on top of each node indicates the bootstrap values of 1000 replications. The peak spectral absorption values are indicated within parentheses. For the accession numbers and citations for the opsin sequences used in the phylogenetic tree construction see Table 2. LWS, long-wavelength sensitive; MWS, middle-wavelength sensitive; SWS, short-wavelength sensitive.

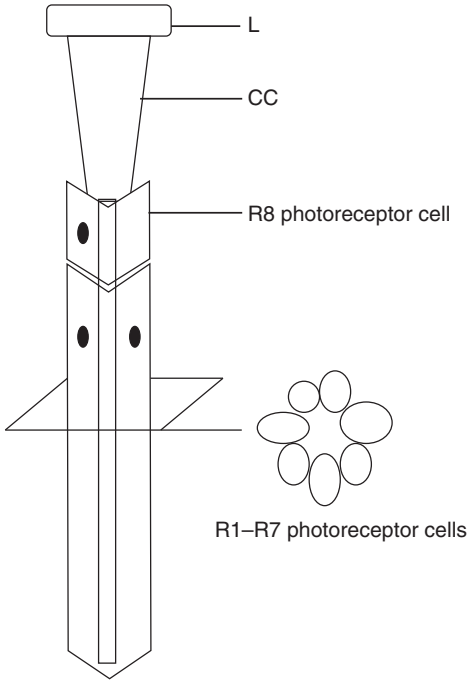

Fig. 4. Schematic of an ommatidium in *U. pugilator*. The lens (L), crystalline cone (CC) and photoreceptor cell (R1–R8) regions are indicated, with the eighth cell located distal to cells R1–R7. A cross section of the ommatidium indicates the arrangement of R1–R7.

subsequently detected using Fast Red tablets (Roche Applied Sciences) in accordance with manufacturer instructions after a brief 10 min wash in 1× PBX at RT.

RESULTS

Identification of opsin genes from *U. pugilator*

Three different opsin cDNAs of 1358, 1467 and 1459 bp were identified by RT-PCR using degenerate and gene-specific opsin primers (Table 1) and named *UpRh1*, *UpRh2* and *UpRh3*, respectively (Fig. 1; GenBank accession numbers: *UpRh1*, HM765425; *UpRh2*, HM765426; *UpRh3*, HM765427). Opsin cDNAs differed in the length of their 5′ and 3′ untranslated regions and in nucleotide sequence conservation, with the 3′ end of each transcript in particular showing low (28%) sequence similarity.

Opsin amino acid sequences were deduced from the full-length cDNA sequences for each opsin. *UpRh1* and *UpRh2* each encode 377 amino acid opsins and *UpRh3* encodes a 376 amino acid opsin. UpRh1 and UpRh2 are 76.72% identical and 89.12% similar in their amino acid sequences, and show 50.13% amino acid identity and 70.13% similarity to UpRh3. As is typical for opsins, all three *U. pugilator* opsins are predicted to be seven-transmembrane-domain proteins. In addition they show strict conservation of (1) the potential G-protein binding regions (QAKKMNV and DRY) (Gartner and Towner, 1995; Townson et al., 1998), (2) the lysine (K) amino acid responsible for covalent binding to the chromophore through a Schiff's base interaction (Gartner and Towner, 1995; Townson et al., 1998; Wang et al., 1980), (3) two conserved cysteine (C) residues (UpRh1, C128 and C205; UpRh2, C128 and C205; UpRh3, C132 and C209) responsible for disulfide linkage stabilization (Gartner and Towner, 1995; Townson et al., 1998) and (4) an aspargine (N) that is a potential glycosylation site (UpRh1 and UpRh2, N3; UpRh3, N9) (Gartner and Towner, 1995; Townson et al., 1998).

Several characteristic amino acids have been identified at specific positions that are functionally related to the spectral sensitivity of the opsins. For instance, a functional, conserved residue at position 131 in the third transmembrane domain of invertebrate opsins is thought to be one of the two negative counter ions stabilizing the protonated Schiff's base (Chang et al., 1995), and in all long-wavelength-sensitive opsins, this residue is a tyrosine (Y), whereas in all short-wavelength opsins, this residue is phenylalanine (F). UpRh1 and UpRh2 contain a tyrosine residue (Y131 and Y131) whereas UpRh3 has a phenylalanine (F134; Fig. 2) residue corresponding to that position. UpRh3 also contains a conserved lysine (K111) that is unique to all insect short-wavelength opsins. In contrast, UpRh1 and UpRh2 have a conserved serine (S142) found in all opsins except the short-wavelength opsins (Fig. 2). Together, these data predict that UpRh1 and UpRh2 are long-wavelength-sensitive opsins, and *UpRh3* is a short-wavelength-sensitive opsin.

To further test these predictions, we determined the phylogenetic relationships of the *U. pugilator* opsins to other arthropod opsins of known sensitivity (Fig. 3). UpRh1 and UpRh2 are nested within the middle-wavelength opsins. In particular, UpRh1 is 91% identical

to *U. vomeris* opsin1 and 84% identical to *H. sanguineus* BCRH1 opsin, the latter has an estimated maxima at 480 nm (Sakamoto et al., 1996). UpRh2 is 87% identical to *U. vomeris* opsin2 and 91% identical to *H. sanguineus* BCRH2 opsin, also sensitive to light at 480 nm (Sakamoto et al., 1996). UpRh3, by contrast, is only 50% identical to the opsins identified from *U. vomeris* and *H. sanguineus.* It is more closely related to short-wavelength opsins (SW), with 71% similarity to ultraviolet-sensitive opsins from *B. impatiens* (absorption maxima unknown) and *A. mellifera* (absorption max. 353 nm), respectively.

Expression of *U. pugilator* opsins in the photoreceptor cells

The retina of *U. pugilator* contains ~8000 nearly identical subunits called ommatidia (Smolka and Hemmi, 2009), each containing eight photoreceptor cells whose microvilli make up a fused rhabdom. In brachyurans, one of these photoreceptors is small and is situated at the distal ends of the cell bodies of the remaining seven photoreceptors. The rhabdomere of this small retinula cell, R8, is found only at the distal tip of the rhabdom in every ommatidium (Marshall et al., 1999). The remaining seven photoreceptors in *U. pugilator* have different sized and shaped cell bodies (Fig. 4). To determine the expression patterns of the *U. pugilator* opsin genes, we performed *in situ* hybridizations on wax sections of randomly oriented eyes. Antisense riboprobes were synthesized from regions with high nucleotide sequence specificity for each opsin to identify transcript-specific expression. No staining was detectable with the sense riboprobes (data not shown).

The mRNAs for all three *U. pugilator* opsin genes are present in every ommatidium in the eye, with no apparent spatial differences between ommatidia from different eye regions. However, opsin-specific differences in expression were observed between photoreceptor cells within individual ommatidia. *UpRh3*, for instance, was restricted to the small R8 cell at the distal end of the photoreceptor portion of each ommatidium (Fig. 5), whereas the putative middle-wavelength opsins are present in the proximal cells R1–R7. *UpRh1* mRNA was found in five of these seven cells (Fig. 6), and *UpRh2* mRNA was found in three of these cells (Fig. 7).

Coexpression of middle-wavelength-sensitive opsins in a subset of photoreceptors in the proximal retina

Since seven retinula cells form the proximal ommatidia, our observation that *UpRh1* and *UpRh2* were expressed in five and three proximal photoreceptors respectively indicates that these genes must be coexpressed in one of the retinula cells. To better define the expression patterns of these related genes, we performed double *in situ* hybridization. The results confirmed those from single *in situ* hybridization, that these two genes share overlapping expression in one of the seven proximal photoreceptor cells (Fig. 8). Fig. 8A shows seven photoreceptor cells, five of which express *UpRh1* and three express *UpRh2*. Expression patterns of mRNA for the latter three using a riboprobe specific to *UpRh2* show that in a single ommatidium only these three photoreceptor cells express *UpRh2* (Fig. 8B). Comparison of several such image pairs indicates coexpression of *UpRh1* and *UpRh2* opsins in a single photoreceptor cell.

DISCUSSION

Three sequentially distinct, full-length cDNAs of opsin genes were identified from *U. pugilator* retinas. By molecular analysis, *UpRh1* and *UpRh2* are predicted to be middle-wavelength sensitive, and *UpRh3* is predicted to be short-wavelength sensitive. These data are consistent with previous behavioral tests and the physiological tests

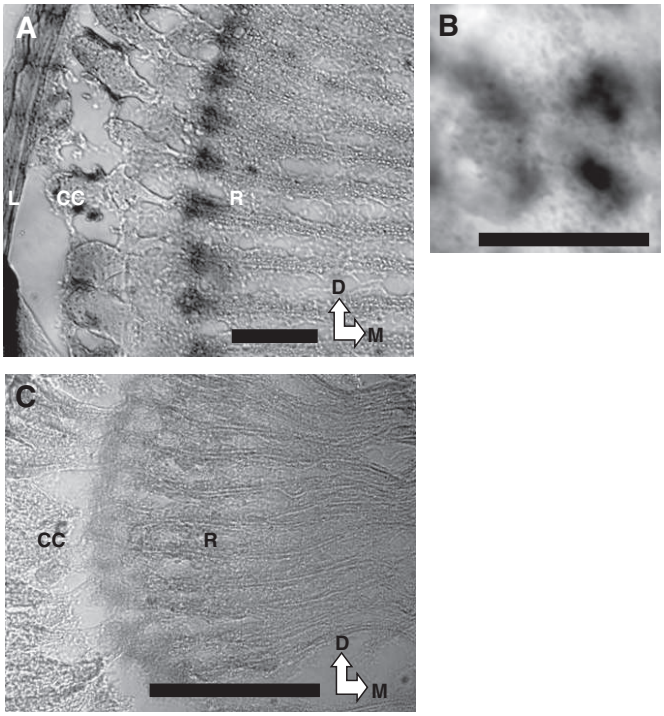

Fig. 5. Expression pattern of *UpRh3* mRNA as determined by *in situ* hybridization. (A) A sagittal view of many ommatidia. Note that *UpRh3* mRNA is present in only a single cell at the distal part of the ommatidium. Scale bar: 50 μm. (B) A cross-section of an ommatidium in which a single four-lobed photoreceptor cell is seen at the distal tip of the ommatidium (R8 cell). Scale bar: 10 μm. (C) A sagittal view of many ommatidia stained with the UpRh3 sense probe as control. No clear staining pattern is seen in any of the ommatidium. Scale bar: 100 μm. CC, Crystalline cone; D, dorsal; L, lens; M, medial; R, photoreceptor cells.

of Hyatt (Hyatt, 1974) and Horch et al. (Horch et al., 2002). The uniform expression pattern in ommatidia across the retina would seem to indicate a similarly uniform sensing capacity, but detailed studies on the physiological effect of screening pigments, and sensitizing pigments, if there are any, are required to determine whether there is any physiological heterogeneity between ommatidia.

Spatial distribution of *U. pugilator* opsins

The results from *in situ* hybridization show that all ommatidia of *U. pugilator* are composed of at least four types of photoreceptor cells that are unique in their spectral sensitivity, thereby satisfying the most fundamental requirement for color vision. This uniform expression pattern is somewhat surprising, since the dorsal and ventral visual fields of semi-terrestrial crabs are probably as consistently exposed to different light environments as any terrestrial animal, because of a highly efficient multi-sensory control system for maintaining eyestalk pitch and roll (Nalbach et al., 1989a; Nalbach et al., 1989b; Nalbach, 1990). However, with only three opsin genes, color vision in all directions probably requires such uniformity of expression. In addition to color discrimination, polarization sensitivity is common in several marine organisms, and its role is similar to color vision in enhancing contrast and conspicuousness, intraspecific signaling, and object identification (Cronin et al., 2003; Glantz and Schroeter, 2006; Glantz, 2008). In fiddler crabs, polarization sensitivity can apparently be used as a celestial direction cue for orienting to the

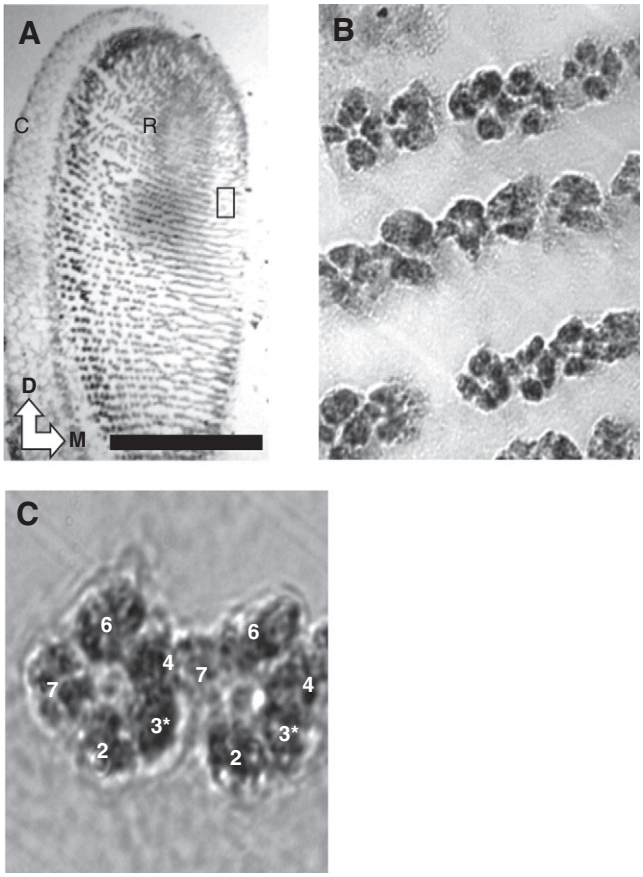

Fig. 6. Expression patterns of *UpRh1* mRNA as revealed by *in situ* hybridization. (A) Uniform expression of *UpRh1* can be seen throughout the retina (R) using Fast Red. C, cone; D, dorsal; M, medial. Scale bar: 500 μm. (B) Enlargement of the boxed region in A. (C) A cross-sectional view of two ommatidia in which expression of *UpRh1* mRNA is present in five of the seven photoreceptor cells (R1–R7), as indicated by the numbers on the two ommatidia. The asterisk indicates the photoreceptor cell that shows coexpression of opsins.

crabs' home beach (Herrnkind, 1968; Chiussi and Diaz, 2002), although it appears to play no role in finer-scale navigation by path integration (Layne et al., 2003). Typically in insects, the dorsal rim area contains UV-sensitive photoreceptors which are also polarization sensitive, coinciding with the fact that natural light tends to be more polarized at shorter wavelengths. UpRh3, the opsin in the most distal cell in each ommatidium, is UV-sensitive and may likewise mediate polarization sensitivity, but this must be done in conjunction with at least one other cell, normally within the same ommatidium. Since the other cells in the ommatidium are not UV sensitive, there is potential for confounding the e-vector orientation and wavelength.

Expansion of ancestral middle-wavelength-sensitive opsin

Based on their peak sensitivity to light, opsins are often classified into three groups: short-wavelength-sensitive opsins (SWS, ultraviolet–blue), middle-wavelength-sensitive (MWS, blue–green) and long-wavelength-sensitive (LWS, green–red). It is thought that the visual system of ancestral insects was trichromatic, with opsins sensitive to UV, blue and green (Briscoe and Chittka, 2001), and in some groups an expansion of the green opsin added red sensitivity, followed by the loss of the blue opsin (Jackowska et al., 2007). Although much less is known of the molecular evolution of crustaceans, crabs do not appear to follow this scheme. They are thought to descend from ancestors with two opsins, one sensitive to violet–ultraviolet, and the other sensitive to blue–green or green (Porter et al., 2007). Among the three opsins identified from the photoreceptor cells of *U. pugilator,* UpRh3 is predicted to be UV sensitive, and UpRh1 and UpRh2 to be MW sensitive. Although most other crustaceans possess an opsin sensitive to considerably longer wavelengths (~500–530 nm), the only brachyuran crab whose opsin gene sequence and spectral sensitivity are both known, *Hemigrapsus sanguineus*, has two MWS opsins, both with peak sensitivity around 480 nm (Sakamoto et al., 1996). The highly similar fiddler crab opsins UpRh1 and UpRh2 can be predicted to have a similar peak, and are likely to be the result of an expansion event of the ancestral MWS opsin. The functional significance of this ostensible bifurcation of the MWS opsin as an adaptation in the color system of semi-terrestrial animals should be further examined in the context of their particular light environment.

Coexpression of opsins in a single photoreceptor cell

The results from the double *in situ* hybridization reveal that the probes for the two opsins, UpRh1 and UpRh2, both label a single photoreceptor cell in each ommatidium. The colocalization of these two probes in a single photoreceptor cell demonstrates that the cell expresses two opsin visual pigments. The once commonly held notion that a single photoreceptor cell expresses only one functional visual pigment has been dispelled many times over, as evidence from both vertebrates and invertebrates has shown this to be an over-generalization. The expression of multiple opsins in a single photoreceptor cell has been reported in mice (Applebury et al., 2000; Lukats et al., 2002; Lyubarski et al., 1999), hamsters (Lukats et al., 2002), rabbits and guinea pigs (Röhlich et al., 1994), fish (Archer and Lythgoe, 1990; Wood and Partridge, 1993; Hope et al., 1998), salamanders (Makino and Dodd, 1996), butterflies (Kitamoto et al., 1998; Arikawa et al., 2003; Sison-Mangus et al., 2006), flies (Mazzoni et al., 2008), beetles (Jackowska et al., 2007), and several

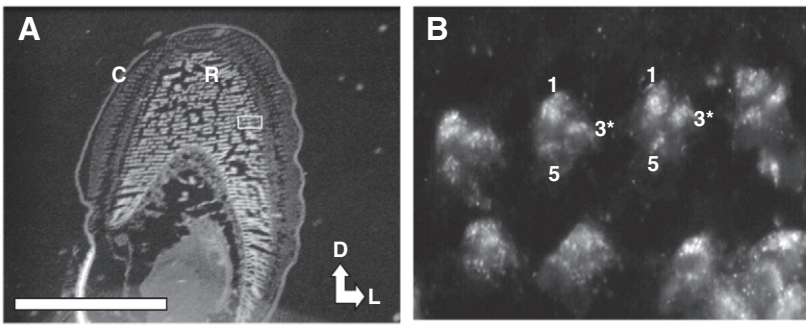

Fig. 7. Expression patterns of *UpRh2* mRNA as revealed by *in situ* hybridization. (A) Uniform expression of *UpRh2* can be seen throughout the retina using Fast Red fluorescence imaging. Scale bar: 500 μm.
(B) Enlargement of the boxed region in A showing a cross-sectional view of ommatidia in which expression of *UpRh2* mRNA is present in three of the seven photoreceptor cells (R1–R7), as indicated by the numbers on the two ommatidia. C, cone; D, dorsal; L, lateral; R, photoreceptor cells.

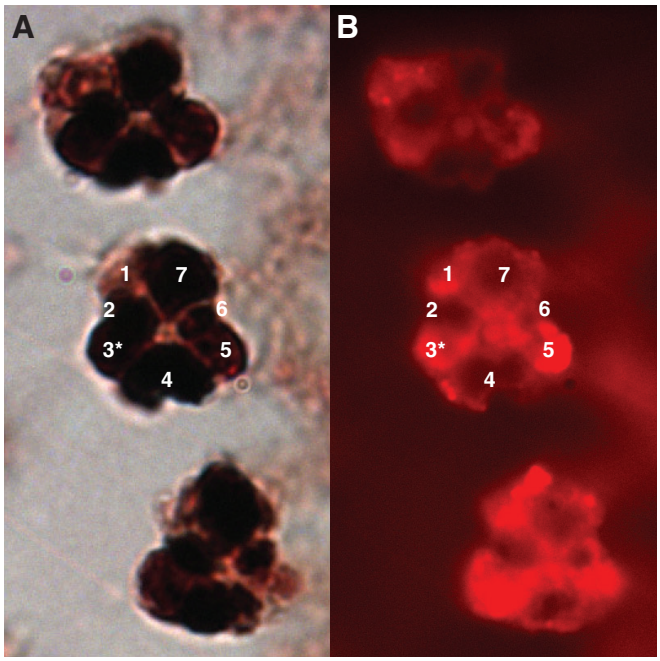

Fig. 8. Distribution of *UpRh1* and *UpRh2* mRNA as revealed by *in situ* hybridization. (A) Double colorimetric staining of ommatidia with riboprobes specific to *UpRh1* (black) and *UpRh2* (red). (B) Fluorescent staining of the same ommatidia with Fast Red specific to *UpRh2*. Note that *UpRh2* mRNA is present in only three cells. The numbers on the ommatidium indicate the seven photoreceptor cells (R1–R7) as seen from a cross-sectional view. The asterisk indicates the photoreceptor cell that is labeled by both the opsin probes.

distantly related crustaceans (Sakamoto et al., 1996; Oakley and Huber, 2004; Frank et al., 2009). In fact, coexpression of opsins in single photoreceptors may even be unusually common in crustaceans (Cronin and Porter, 2008).

The coexpression of opsins now seems to be a fairly common phenomenon, but its functional significance is still a matter of speculation, although at least two reasonable theories exist. The first theory proposes a sensitivity enhancement function, and no role in color vision. Multiple opsin expression causes photoreceptor cells to have a broader spectral sensitivity and, presumably, increases the absolute sensitivity of the cell. These cells may therefore mediate achromatic vision in diurnal invertebrates under low light conditions, similar to rods in vertebrate scotopic vision, a function that has also been proposed for opsin coexpression in murine cones (Applebury et al., 2000). The second theory proposes a role in color vision. If the absorbance spectra of solo-expressed visual pigments are fairly similar, a 'broad-band' cell may be useful for spectral tuning. The two similar signals can be better discriminated by sharpening and amplifying their peaks, essentially by multiplication of the signal from the broad-band cell with the respective single peak signals, thereby providing the animal with finer color discrimination. A variation of this theory that does not strictly involve color perception is that the activity of a broad-spectrum photoreceptor is compared to that of a narrower UV photoreceptor to judge the ratio between UV and all longer wavelengths, which may be useful for orientation (Mazzoni et al., 2008). Although the specific absorbance curves to support this theory have yet to be demonstrated, we speculate that, of the two theories, the opsins expressed in *U. pugilator* conform better to the latter, color perception theory. Unlike, for instance, lycaenid butterflies which have a photoreceptor type coexpressing both a short and a long-wavelength opsin, probably conferring an extremely broad-sensitivity spectrum (Sison-Mangus et al., 2006), the two coexpressed opsins in fiddler crabs fall within the same spectral class. This means that the broadness of their combined spectral absorbance is narrower than it potentially could be, limiting its effectiveness for improving absolute sensitivity and potentially introducing an additional need for signal sharpening.

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