The Journal of Experimental Biology 204, 3333–3344 (2001)
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JEB3527

The molecular basis for spectral tuning of rod visual pigments in deep-sea fish

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Accepted 2 July 2001

Summary

Most species of deep-sea fish possess of a rod-only retina with a pigment that is generally shortwave shifted in λ_{max} towards the blue region of the spectrum. In addition, the λ_{max} values of different species tend to cluster at particular points in the spectrum. In this study, the rod opsin gene sequences from 28 deep-sea fish species drawn from seven different Orders are compared. The λ_{max} values of the rod pigments vary from approximately 520 nm to <470 nm, with the majority lying between 490 nm and 477 nm. The 520 nm pigment in two species of dragon fish is associated with a Phe261Tyr substitution, whereas the shortwave shifts of the pigments in the other 26 species are accountable by substitutions at a further eight sites (83, 122, 124, 132, 208, 292, 299 and 300). Clustering of λ_{max} values does not, however, involve a common subset of these substitutions in the different species. A phylogenetic analysis predicts that the pigment in the ancestral species would have had a λ_{max} of approximately 480 nm. A total of 27 changes is required to generate the pattern of substitutions seen in the different species, with many sites undergoing multiple changes.

Key words: opsin, visual pigment, rod photoreceptor, deep-sea fish, spectral tuning, evolution.

Introduction

The ambient light of the deep-sea is composed of dim blue downwelling daylight and bioluminescence (Marshall, 1979). However, the intensity of downwelling light diminishes rapidly with increasing depth, and the limit of scotopic vision has been calculated to be at about 1000 m in the clearest tropical oceans (Denton, 1990). The visual systems of deep-sea fish show numerous adaptations to this photon-limited visual environment, including the loss of cone photoreceptors to give a rod-only retina, an unusually high amount of visual pigment in the photoreceptors (Denton and Warren, 1957; Partridge et al., 1988; Partridge et al., 1989), large photoreceptor size (Munk, 1966), and the wavelength of maximal absorbance of visual pigments shortwave (SW)-shifted from around 500 nm of the majority of rod visual pigments to around 480 nm to match the wavelength of maximal spectral transmission of oceanic waters (Denton and Warren, 1957; Muntz, 1958). This SW-shifted sensitivity also correlates well with the blue bioluminescence emitted by photophores present on many species of deep-sea fish (Nicol, 1969; Herring, 1983; Douglas et al., 1998b).

Vertebrate visual pigments are composed of an opsin protein of approx. 350 amino acid residues that forms seven α-helical transmembrane regions connected by cytoplasmic and luminal loops (Dratz and Hargrave, 1983; Findlay and Pappin, 1986), covalently attached *via* a protonated Schiff-base linkage to a chromophore. Each visual pigment shows a peak of maximal absorbance (λ_{max}), the precise location depending on interactions between the chromophore and specific amino acid residues of the opsin protein. In a previous study of visual pigments in four species of deep-sea fish with rod-only retinas containing visual pigments with λ_{max} values ranging from 483 nm to 468 nm (Hope et al., 1997), a number of candidate amino acid substitutions for spectral tuning were identified. However, the small number of species studied precluded a more detailed analysis of the mechanism of spectral tuning. A feature of deep-sea fish rod pigments is that their λ_{max} values tend to cluster at particular points in the spectrum rather than forming a continuous distribution (Bridges, 1965; Dartnall and Lythgoe, 1965; Partridge et al., 1989). The molecular basis for this phenomenon is unknown but could clearly be dependent on a common set of amino acid replacements, where each discrete shift in λ_{max} is achieved in all species by the same amino acid substitution.

In order to examine this and to determine whether the mechanism of spectral tuning proposed by Hope et al. (Hope et al., 1997) is of general applicability to the rod visual pigments of deep-sea fish, we have now extended the analysis to a much larger group of species drawn from seven different Orders of the Euteleostei, the Aulopiformes, Beryciformes, Gadiformes, Myctophiformes, Ophidiiformes, Osmeriformes and Stomiiformes. With only two exceptions, all the species studied possess only a single rhodopsin pigment in the retina

(Ali and Anctil, 1976; Fröhlich et al., 1995; Partridge et al., 1988; Partridge et al., 1989; Douglas et al., 1995), indicating that only a single rod opsin gene is expressed in the photoreceptors. The exceptions are two species of dragon fish, *Aristostomias tittmanni* and *Malacosteus niger*. These species emit bioluminescent light with maxima beyond 700 nm (Widder et al., 1984), in addition to the more usual blue light. *M. niger* possesses a rhodopsin/porphyropsin pigment pair with λ_{max} values of 517 nm and 550 nm, respectively. These pigments are based on a single rod opsin gene but with retinal or 3,4-dehydroretinal, respectively, as chromophore (Bowmaker et al., 1988; Douglas et al., 1998b). In addition, however, *M. niger* uses a remarkable photosensitizer based on a mixture of defarnesylated and demetallated derivatives of bacteriochlorophylls *c* and *d* in the retina to enhance the sensitivity of the 'pigment pair' to its own longwave (LW) radiation (Bowmaker et al., 1988; Douglas et al., 1999a). The closely related species, *A. tittmanni*, lacks the photosensitizer. Instead, as well as possessing a rhodopsin/porphyropsin 'pigment pair' with λ_{max} values of 523 nm and 551 nm, it has a third pigment with a λ_{max} of 581 nm, based most probably on a second opsin protein.

Materials and methods

Collection of samples

Deep-sea fish were caught from the NERC research ship RRS Challenger during cruises 113 in 1994 and 122 in 1995, by deep trawling with either a semi-balloon otter trawl (Merrett and Marshall, 1981) or a rectangular midwater trawl combination net, from depths between 600 m and 5000 m, in the area of the Porcupine Sea Bight abyssal slope region (49 °27′N, 11 °29′W to 49 °59′N, 13 °12′W) or the Tagus and Horseshoe abyssal plains (31 °15′, 17 °00′ to 38 °45′, 12 °10′) of the North Eastern Atlantic. Fish were also collected from the western Atlantic in the region of the Bahamas and South Coast of South Carolina, using a similar midwater trawl on two cruises of the R/V Edwin Link (Harbor Branch Oceanographic Institute, Florida, USA). All fish were dead when brought to the surface. Dissected tissue samples or whole fish were either rapidly cooled to −80 °C for storage or placed in absolute ethanol prior to storage at −20 °C.

Sequencing of the rod opsin gene

The region of the rod opsin gene that encodes the seven α-helices and associated cytoplasmic and luminal loops was amplified by polymerase chain reaction (PCR) and sequenced. Genomic DNA was extracted from liver or whole body samples by a standard phenol/chloroform extraction protocol. The first-round PCR amplifications utilised an oligonucleotide primer pair Frho91F (5′-CATATGAATACCCTCAGTACT-ACC-3′) and Frho956R (5′-CCATTACCCATGTAAATG-CAATTCCTG-3′) that amplifies a fragment from nucleotides 91 to 956. Where an amplified product was not visible on an agarose gel, a second nested PCR was carried out using primer pair Frho173F (5′-*TGTAAAACGACGGCCAGT*CTTCCCY-RTCAACTTCCTCAC-3′) and Frho913R (5′-*CAGGAAAC-AGCTATGACC*TGCTTGTTCAWGCAGATGTAG-3′). The 5′ region of the gene was amplified using primer pair Frho16F (5′-WWWWWATGAACGGYACRGAGG-3′) and Frho229R (5′-AGAGGTYRGCMACNGCCAGGTTSAG-3′).

Each PCR contained approximately 100 ng of template DNA, 12.5 μmol l^{-1} of each primer, 0.2 mmol l^{-1} each of dATP, dCTP, dGTP and dTTP, 4 mmol l^{-1} Mg_2Cl, 0.5 unit of *Taq* polymerase and 5 μl of reaction buffer in a final volume of 50 μl. Following an initial denaturation for 3 min at 94 °C, 35 cycles were used with an annealing temperature of 56 °C, an elongation temperature of 72 °C and a denaturing temperature of 94 °C. PCR products were passed through a Centricon 100 column (Princeton Scientific, Inc.) prior to direct sequencing with the Prism FS dye-deoxy *Taq* terminator kit, and the Prism Dyeprimer FS M13 forward and reverse kits. An ABI Model 373a sequencer was employed to generate the sequence. For each specimen, at least three independent PCR fragments were sequenced.

The rod opsin cDNA sequence was obtained for one species, *Gonostoma elongatum*. mRNA was isolated from eye tissue using the QuickPrep mRNA purification kit (Pharmacia) and cDNA synthesised using Superscript II reverse transcriptase, RNase H, and oligo-dT primer. The 5′ and 3′ ends of the coding sequence were then amplified by the RACE system (Gibco BRL) and the resulting products sequenced as described above.

Phylogenetic analysis

Neighbour-joining (Saitou and Nei, 1987) was used to construct a phylogenetic tree from the opsin gene sequences. The degree of support for internal branching was assessed by bootstrapping with 500 replicates. All computations were carried out with the MEGA computer package (Kumar et al., 1993).

Results

Fish species

Table 1 lists the 28 species of deep-sea fish examined in this study, together with the λ_{max} values of their visual pigments. Three of these species, *Cataetyx laticeps*, *Gonostoma elongatum* and *Hoplostethus mediteranus*, were the subject of a previous study (Hope et al., 1997). By far the largest group of species is from the Stomiiformes, reflecting their relative abundance in the area of the north eastern Atlantic where sampling took place.

Identity of opsin gene sequence

As shown in Fig. 1, the amplified sequences form a single clade with the rod opsins of other species that is quite separate from the rod-like brain opsin identified in *Fugu* (Philp et al., 2000), the rod-like green cone opsins, and the other cone opsins, confirming therefore that it is the rod opsin gene that has been amplified. Some of the bootstrap values for branches in this tree are low, although the key branch that separates the teleost rod opsins from the remaining sequences has a

Table 1. *Classification and λ_{max} of visual pigments of deep-sea fish species*

Species	Suborder	Family	Subfamily	Common name	λ_{max} (nm)*
Superorder: Stenopterygii					
Order: Stomiiformes					
Aristostomias tittmani	Photichthyoidei	Stomiidae	Malacosteinae	Loosejaws	523/551/581
Malacosteus niger	Photichthyoidei	Stomiidae	Malacosteinae	Loosejaws	517/542
Photostomias guernei	Photichthyoidei	Stomiidae	Malacosteinae	Loosejaws	483
Chauliodus danae	Photichthyoidei	Stomiidae	Stomiinae	Scaly dragonfishes	484
Chauliodus sloani	Photichthyoidei	Stomiidae	Stomiinae	Scaly dragonfishes	485
Idiacanthus fasciola	Photichthyoidei	Stomiidae	Idiacanthinae	Black dragonfishes	485
Stomias boa	Photichthyoidei	Stomiidae	Stomiinae	Scaly dragonfishes	489
Ichthyocossus ovatus	Photichthyoidei	Photoichthyidae		Lightfishes	489
Vinciguerria nimbaria	Photichthyoidei	Photoichthyidae		Lightfishes	477
Argyropelecus aculeatus	Gonostomatoidei	Sternoptychidae	Sternoptychinae	Marine hatchetfishes	477
Argyropelecus gigas	Gonostomatoidei	Sternoptychidae	Sternoptychinae	Marine hatchetfishes	477
Gonostoma bathyphilium	Gonostomatoidei	Gonostomatidae		Bristlemouths	481
Gonostoma elongatum	Gonostomatoidei	Gonostomatidae		Bristlemouths	483
Superorder: Scopelomorpha					
Order: Myctophiformes					
Diaphus rafinesquei		Myctophidae	Lampanyctinae	Lanternfishes	489
Bolinichthys indicus		Myctophidae	Lampanyctinae	Lanternfishes	489
Ceratoscopelus warmingii		Myctophidae	Lampanyctinae	Lanternfishes	488
Lampanyctus alatus		Myctophidae	Lampanyctinae	Lanternfishes	485
Benthosema suborbitale		Myctophidae	Myctophinae	Lanternfishes	487
Superorder: Paracanthopterygii					
Order: Gadiformes					
Phycis blennoides		Phycidae	Phycine	Phycine hakes	494
Coryphaenoides guntheri		Macrouridae	Macrourinae	Grenadiers or rattails	479
Order: Ophidiiformes					
Bassozetus compresis		Ophidiidae	Ophidiinae	Cusk-eels	476
Cataetyx laticeps		Bythitidae	Bythitinae	Brotulas	468
Superorder: Protacanthopterygii					
Order: Osmeriformes					
Conocara salmonea		Alepocephalidae	Alepocephalinae	Slickheads	480
Alepocephalus bairdii		Alepocephalidae	Alepocephalinae	Slickheads	476
Superorder: Acanthopterygii					
Order: Beryciformes					
Anoplogaster cornuta		Anoplogastridae		Fangtooths	485
Hoplostethus mediteranus		Trachichthyidae		Slimeheads	479
Superorder: Cyclosquamata					
Order: Aulopiformes					
Bathysaurus ferox		Synodontidae	Harpadontinae	Lizardfishes	481
Bathysaurus mollis		Synodontidae	Harpadontinae	Lizardfishes	479

*λ_{max} values were obtained from R. H. Douglas (personal communication), Douglas and Partridge, 1997; Douglas et al., 1999; Partridge, 1989; Partridge et al., 1988; Partridge et al., 1989.

significant value of 97. Additional confirmation comes from the observation that in each case, the amplified gene completely lacked introns, a feature that is limited amongst vertebrate opsins to the teleost rod opsin gene (Fitzgibbon et al., 1995; Hope et al., 1997).

Amino acid sequence of rod opsin

With the exception of the three species from the Gadiformes discussed below, the sequenced region of the gene included the coding region for all seven α-helical regions. The deduced amino acid sequences are shown in Fig. 2. In order to confirm that these genomic sequences correspond to the expressed sequence in the retina, the complete cDNA sequence of one species, *Gonostoma elongatum*, was determined. This sequence is also shown in Fig. 2 and is identical to that obtained previously (Hope et al., 1997) from genomic DNA.

A number of functionally important residues identified by previous studies are conserved across all species. Among these

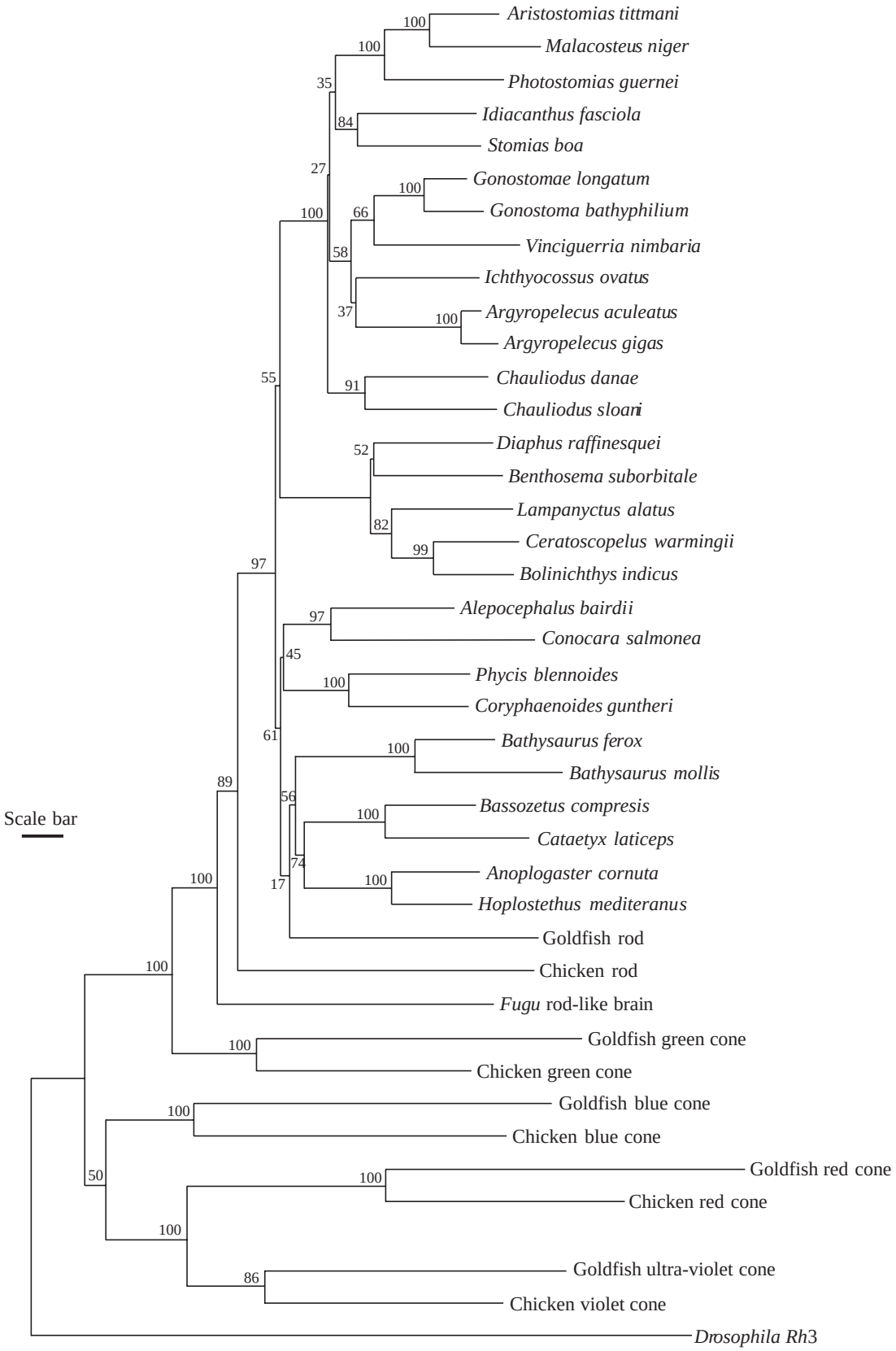

Fig. 1. Phylogenetic tree of opsin gene sequences. The tree was generated by the neighbour-joining method (Saitou and Nei, 1987). The bootstrap confidence values are shown for each branch. The scale bar is calibrated at 0.02 substitutions per site. GenBank accession numbers: *Fugu* rod-line brain AF201472, Goldfish rod L11863, blue cone L11864, green cone l11865 and red cone L11867; chicken rod D00702, violet cone M92039, blue cone M92057, green cone M92038 and red cone X57490, and *Drosophila Rh*3 M17718.

are the Lys296 chromophore attachment site (Dratz and Hargrave, 1983), the Glu113 Schiff base counterion (Sakmar et al., 1989), sites of disulphide bond formation at Cys110 and Cys187 (Karnik and Khorana, 1990), and Trp126 and Trp265 (Nakayama and Khorana, 1990; Nakayama et al., 1998) involved in conformational changes during chromophore isomerisation and formation of the retinal binding pocket. A conserved tripeptide at the cytoplasmic boundary of helix 3 has been shown to be important in transducin binding (Franke et al., 1990). In mammals and birds, this tripeptide is generally Asp/Glu134, Arg135 and Tyr136. In fish, however, Trp is frequently substituted for Tyr at site 136 (Johnson et al., 1993; Hope et al., 1997) and this is seen in five of the six Orders examined here. The exception is the stomiiforms, where Tyr136 is generally present but is replaced by Phe in *Malacosteus niger* and by Trp in *Vinciguerria nimbaria*.

Candidate sites for spectral tuning

Except for species from the same genus, the amino acid divergence between the rod opsin sequences of these deep-sea fish species is generally >20 %, reflecting the diversity of species examined in this study. In order therefore to identify candidate sites for spectral tuning, two overlapping approaches were used. The sequences were mapped on to a model based on conserved residues across >500 G-protein-linked receptor proteins (Baldwin, 1993; Baldwin et al., 1997). This model not only identifies the seven helices but, together with the three-dimensional map of frog rhodopsin determined by electron cryo-microscopy (Schertler and Hargrave, 1995), also provides a framework for orientating each helix with respect to the exterior lipid membrane and the central hydrophilic retinal-binding pocket. Only substitutions that result in a change in either charge or polarity (Nathans, 1990; Nakayama and Khorana, 1991) in residues that are located in the transmembrane helical regions and are either adjacent to this pocket or face another helix appear to be important for the spectral tuning of the resulting pigment (Merbs and Nathans, 1993; Asenjo et al., 1994; Hope et al., 1997; Hunt et al., 1996). The number of candidate tuning sites was then extended to include additional sites identified from the crystal structure of bovine rhodopsin (Palczewski et al., 2000) that are again either adjacent to the retinal binding pocket or the Schiff base. These include part of extracellular loop 2 between helices 4 and 5 that folds deeply into the centre of the molecule, with residues 186–190 contributing to the chromophore-binding pocket. Finally, site 181 was also included, since a Glu181Gln substitution has been shown to result in a 10 nm LW-shift in bovine rhodopsin (Terakita et al., 2000). The Glu at this site is, however, totally conserved across all the deep-sea fish species.

The identity of these residues and their relative position in the helices/loop regions is shown in Table 2. The following analysis of the amino acid sequences of deep-sea fish rod opsins focuses on changes at these sites.

Species from the Order Stomiiformes

Aristostomias tittmani, *Malacosteus niger* and *Photostomias guernei* are all members of the sub-family Malacosteinae (loosejaws). They differ in that *A. tittmani* and *M. niger* are the only species amongst those studied with λ_{max} values >500 nm. *M. niger* possesses a rhodopsin/porphyropsin pigment pair based on a single opsin gene (Bowmaker et al., 1988; Douglas et al., 1999). Comparing the sequence of the *M. niger* gene with

Table 2. *Position in α-helices of amino acid residues that line the chromophore binding pocket or are in close proximity to the chromophore*

	Helix 1														Helix 2							
Position in α-helix	9	10	13	14	17	20	24								5	6	9	12	13	16	20	24
Residue	43	44	47	48	51	54	58								75	76	79	82	83	86	90	94
	Helix 3														Helix 4							
Position in α-helix	8	9	12	13	14	15	16	18	19	20	23	24	26	27	6	7	10	14	17	21		
Residue	114	115	118	119	120	121	122	124	125	126	129	130	132	133	156	157	160	164	167	171		
	Extracellular loop 2																					
Position in loop	8	13	14	15	16	17																
Residue	181	186	187	188	189	190																
	Helix 5														Helix 6							
Position in α-helix	8	9	12	13	16	17	20	23	24						11	12	15	18	19	22	23	25
Residue	207	208	211	212	215	216	219	222	223						257	258	261	264	265	268	269	271
	Helix 7																					
Position in α-helix	7	8	9	10	14	15	17	18	20	21												
Residue	292	293	294	295	299	300	302	303	306	307												

The position of residues in each helix is based on the model of Baldwin (Baldwin, 1993) and the crystal structure of bovine rhodopsin (Palczewski et al., 2000).

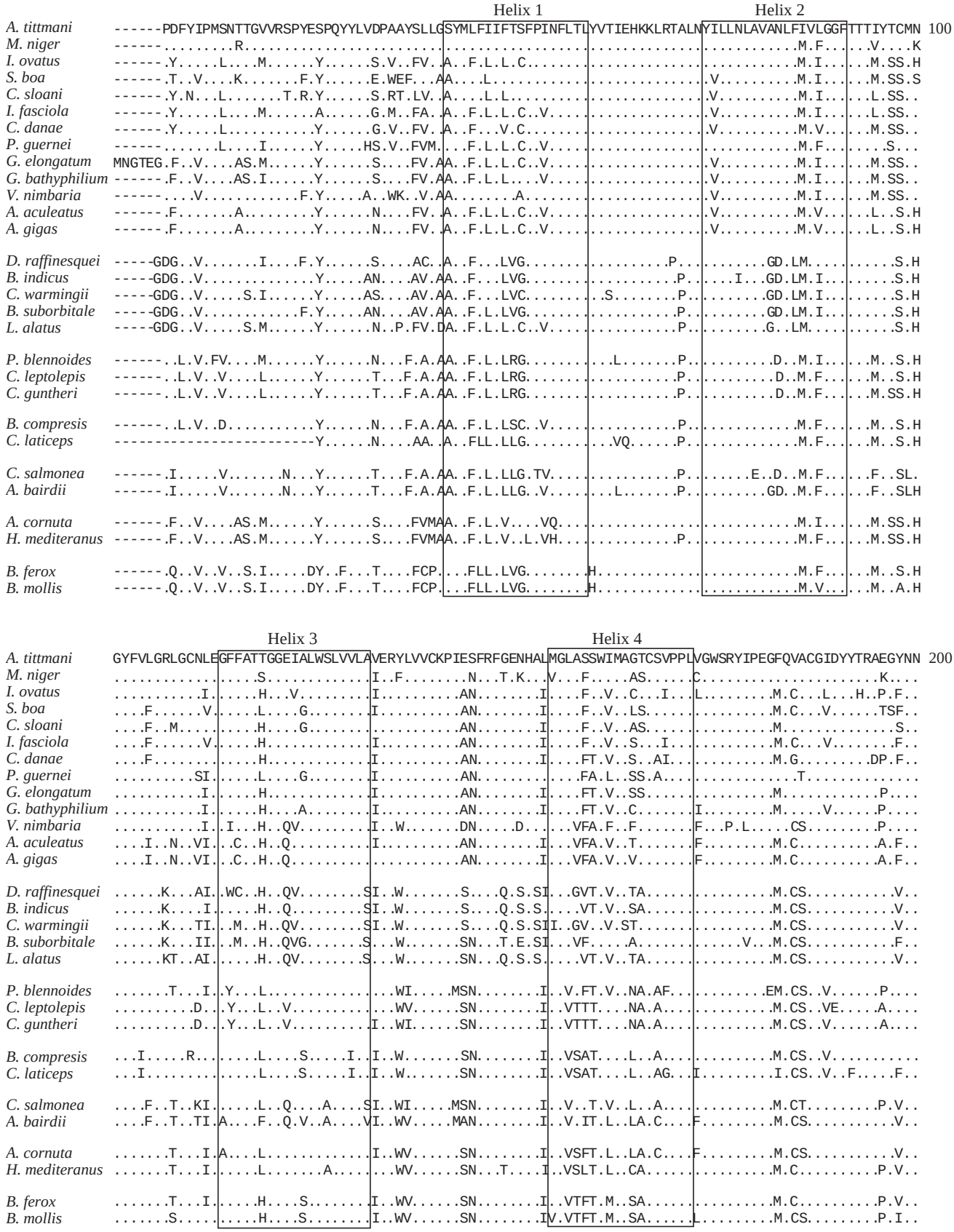
Helix 1
Helix 2
Helix 3
Helix 4
A. tittmani
M. niger
I. ovatus
S. boa
C. sloani
I. fasciola
C. danae
P. guernei
G. elongatum
G. bathyphilium
V. nimbaria
A. aculeatus
A. gigas
D. raffinesquei
B. indicus
C. warmingii
B. suborbitale
L. alatus
P. blennoides
C. leptolepis
C. guntheri
B. compresis
C. laticeps
C. salmonea
A. bairdii
A. cornuta
H. mediteranus
B. ferox
B. mollis

Fig. 2

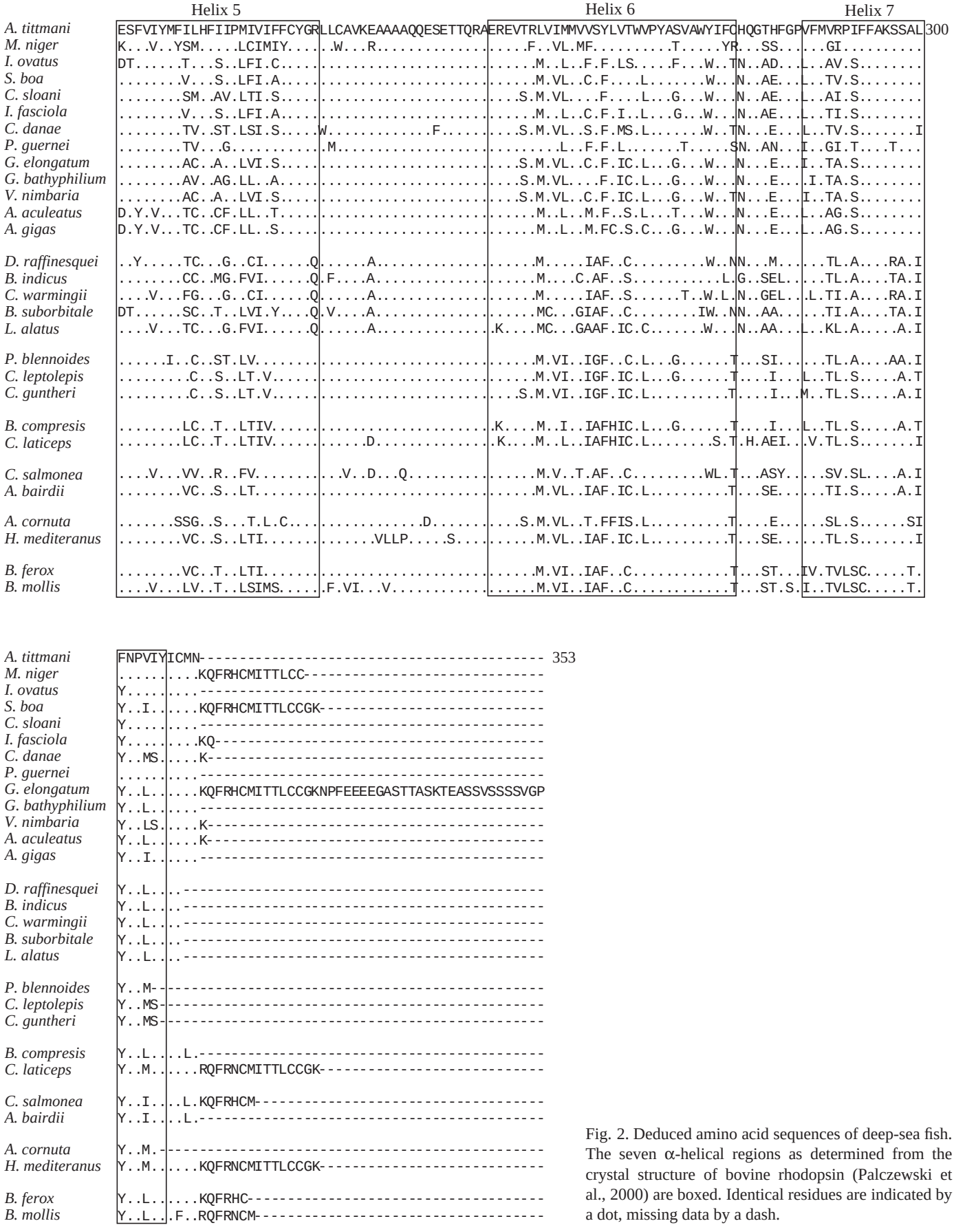

Fig. 2. Deduced amino acid sequences of deep-sea fish. The seven α-helical regions as determined from the crystal structure of bovine rhodopsin (Palczewski et al., 2000) are boxed. Identical residues are indicated by a dot, missing data by a dash.

that of *P. guernei* (pigment λ_{max} of 483 nm), three substitutions at potential tuning sites are apparent: Phe208Tyr in helix 5, Phe261Tyr in helix 6, and Ser292Ile in helix 7. Of these, only the latter two sites have been previously implicated in the tuning of natural pigments. The equivalent of Phe261Tyr is responsible for 6–10 nm of the shift between the primate red and green cone pigments (Asenjo et al., 1994) and Phe261Tyr generated by site-directed mutagenesis resulted in a LW shift of λ nm in the rod pigment of the cave fish, *Astyanax fasciatus* (Yokoyama et al., 1995). Ser292Ala resulted in a shift of 10 nm and 12 nm, respectively, in the rod opsins of bovine (Sun et al., 1997) and the dolphin (Fasick and Robinson, 1998). Both sites have been implicated in the tuning of rod opsins in Baikal cottoids (Hunt et al., 1996).

In contrast to *M. niger*, *A. tittmani* probably possesses two opsin genes. Phylogenetic analysis indicates that the sequence we have obtained is the rod opsin orthologue (see Fig. 1), and a comparison of the deduced amino acid sequence with that of *M. niger* suggests that it would generate a pigment with λ_{max} of not more than 520 nm. The key features are the presence of Tyr261 and Ile292 in both species and the absence of any other modifications such as a chloride-ion binding site (Wang et al., 1993) that would indicate a LW shift to 581 nm. It also lacks Phe208Tyr, identified above as a potential candidate for the LW shift of the *M. niger* pigment, indicating that substitutions at 261 and 292 may together be sufficient to shift the λ_{max} of the pigment in both species to approx. 520 nm, with the substitution at 208 responsible for the 5 nm SW shift of the *M. niger* pigment compared with the *A. tittmani* pigment.

Eight of the stomiiforms, including *P. guernei*, fall into a group with λ_{max} values ranging from 489 nm to 481 nm. Potential tuning substitutions amongst this group are present at sites 51, 160, 186 and 294, but there is no consistent pattern of substitution that would account for the spectral shifts; it is unlikely therefore that any of these substitutions have an effect on spectral tuning. In contrast, species with λ_{max} values <480 nm all possess Glu122Gln. This substitution in bovine and human rod opsins is known to result in a 15–20 nm shortwave shift (Sakmar et al., 1989; Nakayama and Khorana, 1990; Imai et al., 1997), sufficient therefore to account for the shift from 489 nm to 477 nm present in the two *Argyropelecus* sp. and in *Vinciguerria nimbaria*. The latter species differs from the former two at two other sites, 51 and 264, although the identical λ_{max} values of these three species would seem to rule out a role for the substitutions at these sites.

How do these amino acid substitutions fit in with the classification of the Stomiiformes? For the loosejaws *A. tittmani*, *M. niger* and *P. guernei*, the most parsimonious sequence of events is that the Phe261Tyr and Ser292Ile substitutions in the LW-shifted pigments of *A. tittmani* and *M. niger* occurred after the separation of the lineage leading to *P. guernei*, and this is supported by the phylogenetic analysis shown in Fig. 1. The acquisition of the Glu122Gln substitution is a little more complicated. It is present in *A. aculeatus* and *A. gigas* from the family Sternoptychidae, but in only one of the two members of the family Photoichthyidae (*V. nimbaria*). All three species have a λ_{max} at 477 nm. This tuning substitution therefore either occurred separately in the two families, or was lost in the other member of the Photoichthyidae. Phylogenetic analysis of the pattern of substitutions supports the former explanation (Fig. 1).

Species from the Order Myctophiformes

The five myctophid species examined have similar λ_{max} values to the mid-range stomiiforms, that is the group with λ_{max} values of 489–481 nm. They differ from this group, however, at three potential tuning sites, possessing Gln rather than Glu at 122, Ser rather than Ala at 132, and Ala rather than Ser at 292. The substitutions at sites 122 and 292 would be expected to have opposite tuning effects and would account therefore for the positioning of the λ_{max} values of these species in the mid-range. The effect if any of Ser132 is difficult to gauge. *Lampanyctus alatus* differs from the other four myctophid species at site 83; this substitution may account therefore for the small SW shift of *L. alatus* compared to the other myctophid species. There are also differences at site 51 and 264 but, as found for the stomiiforms, these substitutions are found in species with very similar or identical λ_{max} values and are unlikely to be involved in tuning.

Species from the Order Gadiformes

The gadiforms are represented by two species, *Phycis blennoides* and *Coryphaenoides guntheri*. Unfortunately, it proved impossible to extend the 3′ end of the rod opsin sequences in these species up to the end of the transmembrane region of helix 7. The sequence of this region therefore falls short of two candidate sites, 306 and 307 (Fig. 2). These sites are completely invariant, however, in all other species and unlikely therefore to be involved in tuning.

The λ_{max} values for *C. guntheri* is SW-shifted by 15 nm compared to that of *P. blennoides*. These rod opsin gene sequences differ at three candidate tuning sites, 122 where *C. guntheri* uniquely possesses Val, 207 where Met in *C. guntheri* is replaced by Ile in *P. blennoides*, and 292 where Ser in *C. guntheri* is replaced by Ala in *P. blennoides*. This latter substitution is capable of generating a 15 nm shift by itself and may account therefore for the spectral location of the *P. blennoides* pigment at 494 nm. In support of this, a Glu122Ile substitution generated in chicken rod opsin was without effect on spectral tuning (Imai et al., 1997), so it is probable that the similar Glu122Val substitution is also without effect. The effect of the Met207Ile substitution remains uncertain. Finally, the rod opsins of gadiforms differ from those of the stomiiforms in possessing Asp rather than Asn at site 83. In this regard, they are similar to the majority of the myctophids.

Species from the Order Ophidiiformes

The two ophidiiforms are *Bassozetus compresis* and *Cataetyx laticeps*; the rod opsin gene sequence for the latter species was originally reported by Hope et al. (Hope et al., 1997). Both species have SW-shifted λ_{max} values at 476 nm

and 468 nm, respectively. In fact, *C. laticeps* has the shortest λ_{max} of any species examined in this study. Both species possess Ser292, which would account for much of the SW shift, and they also possess Ser124. The additional SW shift of λ nm in *C. laticeps* pigment may be accounted for by the Thr300Ile substitution.

Species from the Order Osmeriformes

The λ_{max} values of the rod pigment in the two osmeriform species, *Conocara salmonea* and *Alepocephalus bairdii*, are 480 nm and 476 nm respectively. Both possess Ser292, which would account for the SW shift of both pigments. They differ at two potential tuning sites, 132 and 168. Site 168 is, however, without effect when substituted in bovine rod opsin (Phyllis Robinson, personal communication); the 4 nm SW shift in the rod opsin of *A. bairdii* is probably therefore the result of the replacement of polar Ser132 by Val.

Species from the Order Beryciformes

The rod visual pigments in two species of beryciforms, *Anoplogaster cornuta* and *Hoplostethus mediteranus*, have λ_{max} values of 485 nm and 479 nm, respectively. The latter species was first studied by Hope et al. (Hope et al., 1997). Ser292 is again present in both species but they differ at site 299, where polar Ser rather than Ala is present in the opsin of *A. cornuta* with the slightly longer λ_{max}.

Species from the Order Aulopiformes

The aulopiforms are represented by two species from the same genus, *Bathysaurus ferox* and *B. mollis*, with similar λ_{max} values of 481 nm and 479 nm, respectively. Both species again possess Ser124 and Ser292, plus Thr rather than Ala at 299.

Evolution of rod opsins

Overall, therefore, substitutions at only nine sites effectively

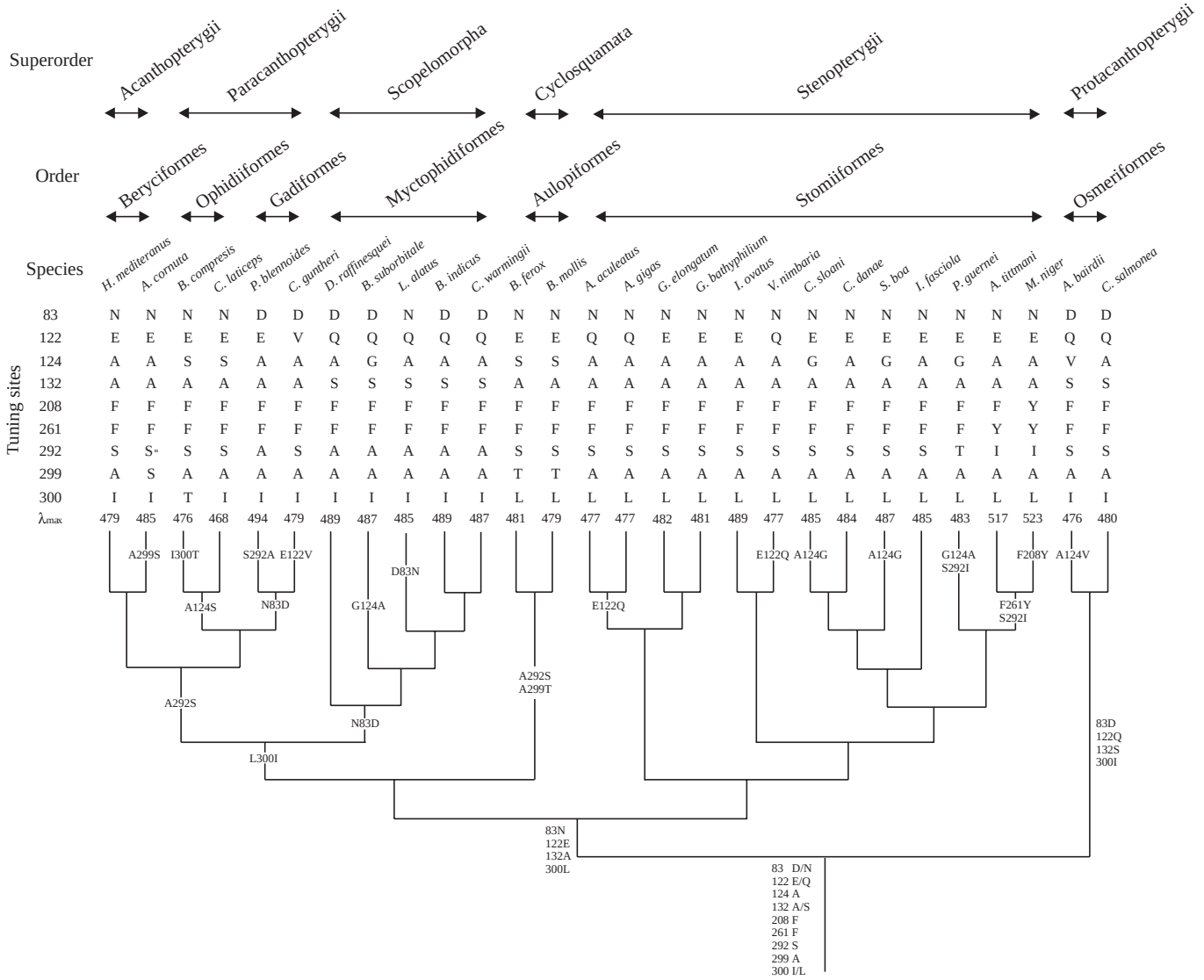

Fig. 3. Phylogeny of deep-sea fish species. The amino acid residues at each of the nine candidate tuning sites are identified and the deduced position within the tree of each substitution is shown.

account for the spectral differences seen in the different species, although the involvement of other sites with small effects cannot be discounted and the mechanism of tuning between 489 nm and 480 nm in the stomiiforms remains to be established. These nine sites are shown in Fig. 3, where they have been placed on to a phylogeny based on a classical cladistic analysis (Nelson, 1995). Of the Superorders examined in this study, the most basal is the Protoacanthopterygii, followed by the Stenopterygii, Cyclosquamata, Scopelomorpha, Paracanthopterygii and Acanthopterygii. The species have been grouped according to family or sub-family divisions as appropriate (Table 1). Where more than two members of a sub-family are present, they have been grouped according to the neighbour-joining (Saitou and Nei, 1987) analysis of the nucleotide sequence of the rod opsin gene presented in Fig. 1.

This phylogenetic analysis allows certain predictions to be made about the amino acid composition of rod opsin in the ancestral species. For five sites, Ala124, Phe208, Phe261, Ser292 and Ala299, an unequivocal assignment can be made, as shown in Fig. 3, whereas for each of the remaining four sites, it is not possible to distinguish between the two alternative residues since the immediate descendants differ at each site. In total, 27 changes are required to generate the pattern of substitutions seen in these species, with many sites undergoing forward and reverse changes on a number of different occasions.

Discussion

The visual systems of deep-sea fish are adapted to the perception of down-welling day light that is spectrally filtered to a narrow band of radiation of between 470 and 480 nm (Kirk, 1983), and to bioluminescent light, which generally peaks around 475 nm (Herring, 1983; Widder et al., 1983; Douglas et al., 1998b). Of the 28 species examined here, all but one express a single rod opsin in the retina, and the amplification of only a single rod opsin gene in each species is consistent with this observation. The exception is the stomiid *A. tittmani*, where at least two retinal visual pigments based on different opsins have been identified spectrophotometrically with λ_{max} values at 523 nm and 581 nm for the A1-based pigments (Partridge and Douglas, 1995). Here again, the sequence that was amplified in this species is clearly also from a rod opsin gene which, on the basis of sequence identity with its most closely related species *M. niger*, would most probably encode the more SW-sensitive pigment.

Candidate spectral tuning sites have been identified by mapping the amino acid sequence of each fish rod opsin onto a three-dimensional model (Baldwin, 1993; Schertler and Hargrave, 1995; Baldwin et al., 1997). Potential tuning sites are identified as those that either point into the chromophore-binding pocket or face other helices. This set of sites was then extended to include all sites shown by Palczewski et al. (Palczewski et al., 2000) to be in close proximity to the chromophore or Schiff base. The stomiiforms form the largest group studied, comprising 13 species with λ_{max} values for their rod opsins that range from 522 nm to 477 nm. Two substitutions, Phe261Tyr and Ser292Ile, appear largely responsible for the shift from the more typical λ_{max} values of between 489 nm and 483 nm to the longer wavelengths of *A. tittmani* and *M. niger* at around 520 nm. The additional shift to below 480 nm can be accounted for by the replacement of charged Glu by uncharged Gln at site 122. What remains unclear from this data set is the mechanism of the smaller shifts in the 489–481 nm region.

When the rod opsin sequences of the fish from the other six Orders of Euteleostei are included alongside the stomiiforms, a further six candidate tuning sites can be identified, making a total of nine sites (Fig. 3). Of these however, only four at residues 83, 122, 124 and 292 are commonly substituted in the different species. Asp83Asn was originally proposed by Hope et al. (Hope et al., 1997) to be one of the main substitutions for the tuning of the rod opsins of deep-sea fish to λ_{max} values of <490 nm, and site 292, which is commonly occupied by polar Ser or Thr, is responsible in most species for a substantial part of the SW shift. The major exception is in the myctophids, where Ala292 is present; in this group, the SW shift is achieved instead by polar Gln122. The SW-shifted pigments of stomiiforms and osmeriforms, with λ_{max} values of 480 nm and below, possess both Gln122 and Ser292. In contrast, similar SW shifts seen in the aulopiforms are achieved by Ser124, rather than Gln122, paired with Ser292.

Dartnall and Lythgoe (Dartnall and Lythgoe, 1965) and Bridges (Bridges, 1965) were the first to note that the λ_{max} values of vertebrate visual pigments cluster around certain points in the spectrum. This is particularly apparent in primate M and L cone pigments, with each spectral location attributable to a particular combination of amino acid residues at three main sites (Neitz et al., 1991; Williams et al., 1992; Hunt et al., 1998; Dulai et al., 1999). The same phenomenon is seen in the rod pigments of deep-sea fish (Partridge et al., 1989; Partridge et al., 1992) and a similar explanation has been advanced, namely that the spectral location of pigments belonging to the same cluster group arises from a common set of amino acid substitutions (Douglas et al., 1998b). The pattern of substitutions across the 28 species included in this study indicates, however, that for the four most commonly used sites, this is only true for species from the same Order. In general, therefore, the presence of cluster points in deep-sea fish rod opsins cannot be attributed to the selection of a particular set of amino acid substitutions. Rather, it is achieved by different combinations of amino acids at these sites that produce the same net spectral shift. This is summarised in Table 3, where representative species from different Orders with the same or very similar λ_{max} values are listed. For each cluster group, there are at least two different combinations of residues at the four sites.

The phylogenetic distribution of amino acid substitutions indicates that the rod opsin of the ancestral species would have possessed Ser292 and that the resulting pigment would have been SW-shifted to around 485 nm or 480 nm, depending on whether Glu or Gln was present at site 122. This implies that species with SW-shifted rod visual pigments were present at or near the base of the Euteleost lineage, adapted therefore to the same visual environment as present-day deep-sea fish.

Table 3. *Amino acid residues at four sites in species from different Orders but with pigments that spectrally locate at three cluster points*

Cluster point		λ_{max} (nm)	Amino acid sites			
			83	122	124	292
1	Stomiiformes	489	Asn	Glu	Ala	Ser
	Myctophiformes	489	Asp	Gln	Ala	Ala
2	Stomiiformes	482	Asn	Glu	Ala	Ser
	Gadiformes	482	Asp	Val	Ala	Ser
	Aulopiformes	481	Asn	Glu	Ser	Ser
3	Stomiiformes	477	Asn	Gln	Ala	Ser
	Osmeriformes	477	Asn	Glu	Ala	Ser
	Ophidiiformes	476	Asn	Glu	Ser	Ser

The λ_{max} of a visual pigment depends on at least two factors. Firstly, the strength of the electrostatic interaction between the Glu113 counterion and the protonated Schiff base is critical; substitutions that increase the strength of this interaction and thereby stabilise the ground state will result in a SW shift, whereas those that reduce it will produce a LW shift (Blatz et al., 1971; Kakitani et al., 1985). Secondly, photoexcitation of the chromophore induces a significant increase in π electron delocalization and a corresponding change in dipole moment, with a shift of net positive charge towards the β-ionone ring upon excitation (Kropf and Hubbard, 1958; Mathies and Stryer, 1976). Interactions with charged, polar or polarizable residues that alter delocalization will lead to a change in the energy difference between ground and excited states. An increase in delocalization will result in a LW shift in the absorbance spectrum, and a decrease in a SW shift. Sites 83, 292, 299 and 300 cluster around the protonated Schiff base and negative counterion. Asp83 in bovine rhodopsin, although not directly involved in interactions with the Schiff base, is important in constraining the position of helices 2, 3 and 4 *via* links with other residues (Palczewski et al., 2000). The Asp83Asn substitution in deep sea fish involves a charge change and may have a consequential effect, therefore, on helix positioning in relation to the chromophore, thereby resulting in a stabilisation of the Schiff base counterion. Ala299 is also involved in an inter-helical constraint to the kinked region of helix 6 that may be disrupted on photoactivation (Palczewski et al., 2000). The other two substitutions (Ser292Ala, Ile/Leu300Thr) will both result in a change in the polar environment of the Schiff base. Sites 122, 124, 132 and 261 are all close to the polyene chain of retinal. The residue at site 122 is involved in the interaction between helix 3 and the β-ionone ring of retinal and is one of only three sites that form the cytoplasmic aspect of the retinal binding pocket (Palczewski et al., 2000). The other two are 261 and 265. Substitutions at site 261 are known to cause spectral shifts in primate red and green pigments (Merbs and Nathans, 1993; Asenjo et al., 1994) and are implicated in this study, whereas Trp265 is totally conserved across all deep-sea fish species. Finally, site 208 is in helix 5, in a position to interact *via* a change in polar group with the β-ionone ring of the chromophore.

The residue at site 122 is known to be important in determining the rate metarhodopsin II decay; in site-directed mutagenesis of chicken rod opsin, Glu122Gln or Glu122Ile produced pigments that decay at a significantly faster rate than wild type (Imai et al., 1997). If this feature extends to deep-sea fish, in addition to its effect on spectral tuning, the Glu122Gln substitution present in the three most SW-shifted stomiiform species, in all five species of mytophids, and in two species of osmeriforms, will also result in a more rapid decay of metarhodopsin II and thereby reduce the amplification of the phototransduction cascade. The Glu122Val substitution present in gadiforms, although lacking an effect on λ_{max}, may also have this effect on phototransduction.

A number of the sites identified in this study have been shown to be involved in the tuning of visual pigments in other species. In particular, substitutions at site 261 play a major role in the tuning of primate MW and LW pigments (Neitz et al., 1991; Williams et al., 1992; Merbs and Nathans, 1992; Asenjo et al., 1994), and substitutions at sites 83 and 292 have been implicated in the SW shifts of rod pigments in different species of cottoid fish in Lake Baikal (Hunt et al., 1996), in bovine (Sun et al., 1997) and in the dolphin (Fasick et al., 1998). Such substitutions are clearly separate events and are examples therefore of convergent evolution, a common feature of opsin gene evolution (Hunt et al., 1998), which must reflect the relatively limited number of sites that can change to give the required spectral shift and still result in a fully functional pigment. Convergent evolution must also be the explanation for the occurrence of repeat substitutions at certain of the key sites identified in the deep-sea rod opsins examined in this study.

We thank the Masters, officers and crew of RRS Challenger and R/V Edwin Link, Professor I. G. Priede for his invitation to join cruise 113, and Drs E. Widder and T. Frank for their invitation to join Harbor Branch cruises on the R/V Edwin Link. The work was supported by project and sea-time grants from the Natural Environment Research Council.

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