

## Eyes with basic dorsal and specific ventral regions in the glacial Apollo, *Parnassius glacialis* (Papilionidae)

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### SUMMARY

Recent studies on butterflies have indicated that their colour vision system is almost species specific. To address the question of how this remarkable diversity evolved, we investigated the eyes of the glacial Apollo, *Parnassius glacialis*, a living fossil species belonging to the family Papilionidae. We identified four opsins in the *Parnassius* eyes – an ultraviolet- (P<sub>gUV</sub>), a blue- (P<sub>gB</sub>), and two long wavelength (P<sub>gL2</sub>, P<sub>gL3</sub>)-absorbing types – and localized their mRNAs within the retina. We thus found ommatidial heterogeneity and a clear dorso-ventral regionalization of the eye. The dorsal region consists of three basic types of ommatidia that are similar to those found in other insects, indicating that this dorsal region retains the ancestral state. In the ventral region, we identified two novel phenomena: co-expression of the opsins of the UV- and B-absorbing type in a subset of photoreceptors, and subfunctionalization of long-wavelength receptors in the distal tier as a result of differential expression of the P<sub>gL2</sub> and P<sub>gL3</sub> mRNAs. Interestingly, butterflies from the closely related genus *Papilio* (Papilionidae) have at least three long-wavelength opsins, L1–L3. The present study indicates that the duplication of L2 and L3 occurred before the *Papilio* lineage diverged from the rest, whereas L1 was produced from L3 in the *Papilio* lineage.

Key words: opsin, photoreceptor, compound eye, insect, colour vision, *Parnassius glacialis*.

### INTRODUCTION

The spectral sensitivity of a photoreceptor is primarily determined by the absorption spectrum of its visual pigment molecules. A visual pigment molecule is a protein, an opsin, that binds a vitamin A-derived chromophore. The absorption spectrum of a visual pigment is strongly dependent on the amino acids in the immediate vicinity of the chromophore, so the expression of multiple opsins in an eye is a good indication that the animal has colour vision. The phylogeny of insect opsins so far identified has revealed three clades, which correspond to the ultraviolet- (UV), blue- (B), and long wavelength (L)-absorbing types. In the basic design, the insect eye expresses three opsins from three clades. For instance, the honeybee *Apis mellifera* has the AmUV, AmB, and AmL opsins expressed in the ommatidia, each containing nine photoreceptors. Seven out of the nine photoreceptors express AmL, but the other two express either AmUV or AmB. According to the expression pattern of AmUV and AmB, three types of ommatidia are evident: one photoreceptor with the UV opsin and one with the B opsin; both with the UV opsin; and both with the B opsin (Wakakuwa et al., 2005). This pattern has been found in a number of insect species, including moths (White et al., 2003), butterflies (Briscoe et al., 2003; Sauman et al., 2005; Zaccardi et al., 2006), and bumblebees (Spaethe and Briscoe, 2005).

Accumulating evidence has indicated that butterfly eyes are extremely diverse in terms of their spectral organization (Arikawa, 2003; Sison-Mangus et al., 2006; Wakakuwa et al., 2006; Awata et al., 2009; Briscoe et al., 2010). For example, swallowtail butterflies *Papilio* sp. have at least three L opsins expressed in the compound eye owing to repeated gene duplication events (Briscoe, 1998; Kitamoto et al., 1998). In the family Pieridae, B opsins are duplicated (Arikawa et al., 2005; Awata et al., 2009). Presumably, the diversity of the eye design reflects the diversity of its evolution and of the lifestyles of the different species. To address questions

concerning how the eye diversity evolved and whether and how it is biologically significant, extensive analyses must be carried out in carefully selected species.

Here, we report our study of the eye of the glacial Apollo butterfly, *Parnassius glacialis*, which belongs to the tribe Parnassiini (subfamily Parnassiinae, family Papilionidae). The tribe Parnassiini is the oldest in the subfamily Parnassiinae, which diverged from other papilionid subfamilies in the Cretaceous period (Omoto et al., 2009). Most *Parnassius* sp. occur in high altitude areas of central Asia, the Himalayas, Western China, and Japan, retaining properties that seem to be adaptive to the cold climate of the glacial period. They have hairy bodies and lay hard-shelled eggs on pebbles or dead twigs instead of on their food plant. The eggs hatch in late winter shortly before the food plants (*Corydalis incisa*, Papaveraceae) sprout and develop very rapidly in the cold weather. Adults appear only in early spring.

We identified four opsins in the retinal mRNA, two of which were duplicated L opsins, indicating that the third L opsin in *Papilio* sp. came into existence after the Parnassiinae diverged. The other two opsins were a UV opsin and a B opsin. We localized the opsin mRNAs in the photoreceptors by histological *in situ* hybridization. We thus found that the dorsal region of the eye retained the basic expression pattern seen in other species, including bees. The ventral region exhibited a complex pattern, including complementary expression of two L opsins, as well as the co-expression of UV and B opsins, which was found for the first time among any species so far studied.

### MATERIALS AND METHODS

#### Animals

We used male adults of the glacial Apollo butterfly, *Parnassius glacialis* (Butler), captured in May around Tsukui Lake, Kanagawa, Japan.

### Cloning

The eyes of *Parnassius glacialis* were isolated and prepared for poly-A RNA extraction using QuickPrep Micro mRNA Purification Kit (GE Healthcare, Chalfont St Giles, UK). From the poly-A RNA, cDNA fragments encoding part of visual pigment opsins were obtained by RT-PCR using degenerate primers based on consensus sequences of lepidopteran opsins. The entire cDNA sequences were then determined by 5'- and 3'-RACE methods. The amplified opsin cDNA fragments were purified using Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad, Hercules, CA, USA) and then cloned using TOPO TA Cloning Kit Dual Promoter (Invitrogen, Carlsbad, CA, USA). The clones were sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit and ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

### Phylogenetic analysis and homology modeling

Complete nucleotide sequences of the coding region predicted from the full-length cDNAs were aligned with other insect opsin cDNAs using ClustalW (<http://www.megasoftware.net/>). Transmembrane (TM) domains were predicted by the TMHMM version 2.0 program (<http://www.cbs.dtu.dk/services/TMHMM/>). Phylogenetic trees based on the nucleotide sequences were reconstructed using Bayesian inference methods (BI) coupled with a Markov chain Monte Carlo algorithm executed in MrBayes 3.1.2 (<http://mrbayes.csit.fsu.edu/>), and also by using the maximum likelihood method (ML) executed in PhyML 3.0 with the General Time-Reversible (GTR) substitution model (<http://atgc.lirmm.fr/phyml/>). The reliabilities of the phylogenetic trees were tested using posterior probabilities based on 500,000 replicate analyses (for BI) or 1000 bootstrap replicates (for ML).

The amino acid sequences of Apollo opsins deduced from the nucleotide sequences were also aligned with the bovine and squid rhodopsins, the three-dimensional (3D) structures of which have been identified (Palczewski et al., 2000; Murakami and Kouyama, 2008). Using the Swiss-PdbViewer software (version 4.01), we measured the distances between the chromophore and amino acids around the chromophore and predicted possible amino acid residues responsible for spectral tuning.

### In situ hybridization

Isolated eyes were immersed in fixative solution (4% paraformaldehyde in 0.1 mol l<sup>-1</sup> phosphate buffer, pH 7.2) and then microwave-irradiated six times for 5 s each (total 30 s). After further incubation in the fixative for 20 min at 4°C, the eyes were dehydrated with a graded series of ethanol followed by 100% terpeneol, and infiltrated with xylene. The eyes were then embedded in Paraplast (Sigma-Aldrich, St Louis, MO, USA) and sectioned at 8–10 µm thickness. Serial sections mounted on a slide were deparaffinized with xylene and rehydrated through an ethanol series. The rehydrated sections were treated sequentially with proteinase K at 37°C for 4 min and then 0.25% acetic acid with 0.1% triethanolamine at room temperature for 10 min prior to hybridization.

We then prepared anti-sense cRNA probes that bound specifically to the four cloned opsin mRNAs: they were PgUV, PgB, PgL2 and PgL3 (see Results). The probes for PgUV and PgB were designed to recognize 1491 and 1569 nucleotides including the coding regions and the UTR, respectively. The probes for PgL2 and PgL3 were designed to be specific for non-coding regions of the 3' terminus because the difference in the coding regions was subtle: probe lengths were 441 and 502 nucleotides, respectively. Probes were labeled with digoxigenin-UTP by *in vitro* transcription using T7- or SP6-RNA polymerase. The labeled probes were denatured at 70°C

for 10 min, then quickly cooled on ice for 5 min, added to hybridization solution (50% formamide, 20 mmol l<sup>-1</sup> Tris-HCl, 0.3 mol l<sup>-1</sup> NaCl, 2.5 mmol l<sup>-1</sup> EDTA, 1 mg ml<sup>-1</sup> yeast tRNA, 10% dextran sulfate, 1×Denhardt's solution, pH 8.0) and finally denatured at 90°C for 5 min. The hybridization solution containing 0.5 µg ml<sup>-1</sup> cRNA probes was applied to the sections and kept at 50°C overnight. The sections were sequentially washed at 50°C in 2×SSC (1×SSC=0.3 mol l<sup>-1</sup> NaCl/30 mmol l<sup>-1</sup> sodium citrate) for 15 min, in 2×SSC with 50% formamide for 2 h, treated with 10 µg ml<sup>-1</sup> RNaseA solution at 37°C for 60 min and then washed three times with the Wash buffer (0.15 mol l<sup>-1</sup> NaCl, 0.1 mol l<sup>-1</sup> Tris-HCl, 0.1% Tween 20, pH 7.5) for 20 min. The hybridized probes were reacted with anti-digoxigenin-AP Fab fragments (Roche, Mannheim, Germany) diluted 1:500 overnight at 4°C. Finally, the labeled probes were visualized using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

### Anatomy

Isolated eyes were prefixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol l<sup>-1</sup> sodium cacodylate buffer (CB; pH 7.3) for 2 h at 20–25°C. After a brief wash with CB, the eyes were postfixed in 2% osmium tetroxide in CB for 2 h at 20–25°C. Following dehydration with a graded series of acetone and infiltration with propylene oxide, the eyes were embedded in Quetol 812 (Nissin EM, Tokyo, Japan). The eyes were cut into 5-µm sections with a diamond knife and observed under a microscope (BX51, Olympus, Tokyo, Japan) without any additional staining.

## RESULTS

### Phylogeny of opsins

We identified four opsin-encoding cDNAs in the poly-A RNA extracted from the retinal homogenate of *Parnassius glacialis*. Phylogenetic analyses showed that the four opsins clustered in three clades of insect opsins: one in the UV clade (Fig. 1A), one in the B clade (Fig. 1B) and two in the L clade (Fig. 1C). The B clade contains both B opsins and violet (V) opsins, and the *Parnassius* opsin in this clade clustered with the *Papilio* B opsin, PxB, peaking at 460 nm (Kinoshita et al., 2006). Based on the clustering pattern, we hereafter refer to the opsins in the UV and B clade as PgUV and PgB (*Parnassius glacialis* UV and B), respectively. The two L types appear to be orthologs of L2 and L3 of *Papilio*, so we refer to the two L opsins of *Parnassius glacialis* as PgL2 and PgL3, respectively, according to the numbering of the *Papilio* L opsins (Fig. 1C). Despite extensive searches for orthologs of the *Papilio* L1 using several different primer sets, we could not find any sign of an L1 ortholog in the eye of *Parnassius glacialis*.

### Localization of opsin mRNAs in photoreceptors

We localized the mRNAs of the four opsins in the retina by histological *in situ* hybridization using four antisense cRNA probes, each binding specifically to one of the four opsin mRNAs. Absence of cross-hybridization of the probes was confirmed by dot blot analysis (data not shown). A diagram of an ommatidium of *Parnassius* is shown in Fig. 2. Each ommatidium has nine photoreceptors, R1–R9, which bear photoreceptive microvilli forming a rhabdom along the central axis. The distal half of the rhabdom consists of the microvilli of four distal photoreceptors, R1–R4, whereas four proximal photoreceptors, R5–R8, form the proximal half of the rhabdom. R9 is the basal photoreceptor that adds a few microvilli at the base of the rhabdom.

In longitudinal sections, PgUV and PgB labeling was detected only in the distal tier of the retina (Fig. 3A,B). PgL2 labeling was

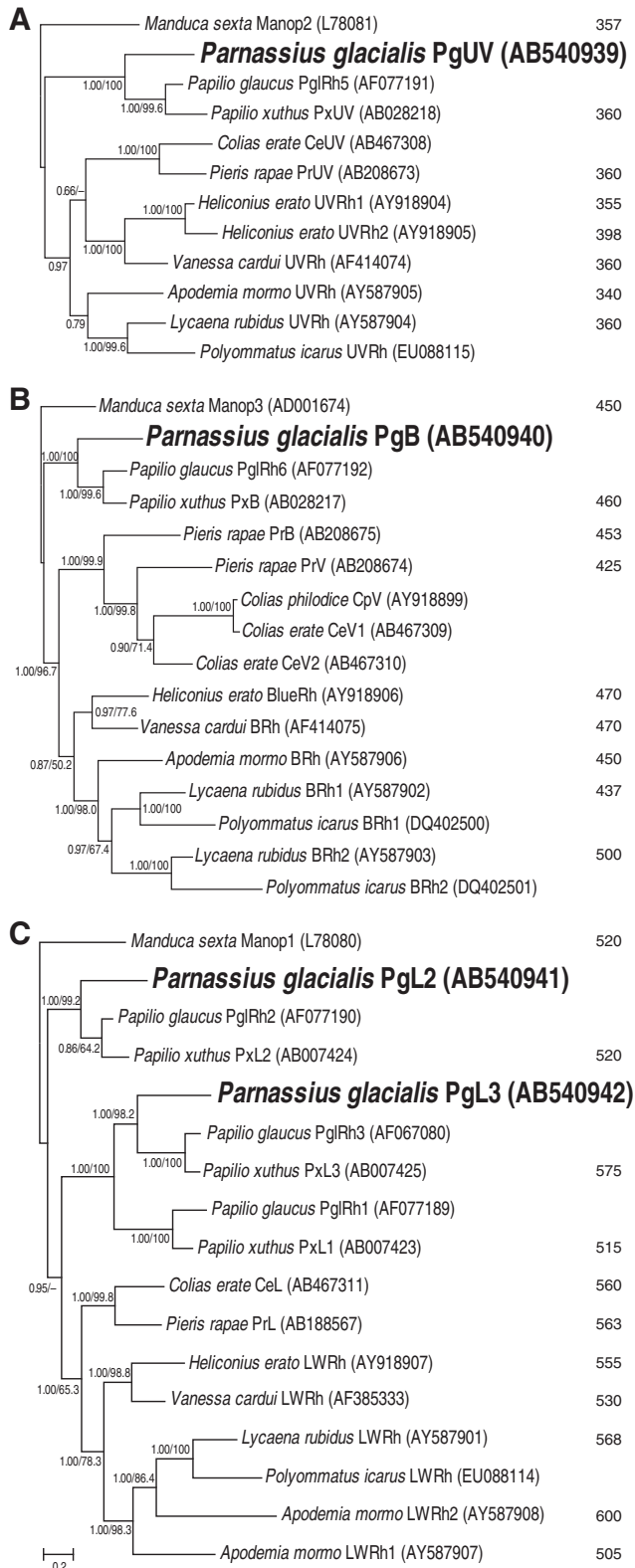


Fig. 1. Phylogenetic relationship of the (A) ultraviolet (UV), (B) blue (B) and (C) long wavelength (L) opsins determined by Bayesian inference analyses based on sequences of 1155 (UV and L) or 1161 (B) nucleotides. ML phylogenetic analyses also produced very similar trees, with only a minor difference in branching of *Apodemia mormo* UVRh clustering with pierid and nymphalid species (not shown). The numbers at the nodes indicate the Bayesian posterior probabilities and ML bootstrap values. Values under 0.5 are not shown.

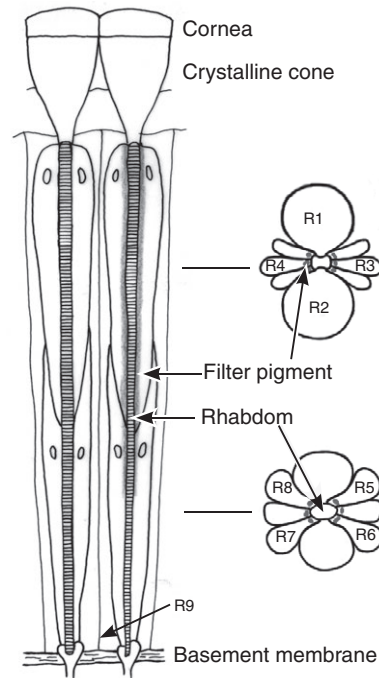


Fig. 2. The ommatidia of *Parnassius glacialis*. The longitudinal views are based on the ventral three types of ommatidia, type I-v (left), type II-v and type III-v (right). Type II-v is devoid of the perirhabdomal filter pigment (see Fig. 5). An ommatidium contains nine photoreceptors, R1–R9, which together form a fused rhabdom in the centre.

detected throughout the eye, but more strongly in the proximal tier (Fig. 3C), whereas Pgl3 labeling was detected only in the ventral three quarters of the eye (Fig. 3D).

We investigated the labeling pattern in detail in serial transverse sections. Figure 3 shows four sets of results: the dorsal region at the distal (Fig. 3E–H) and the proximal (Fig. 3I–L) tier, and the ventral region at the distal (Fig. 3M–P) and the proximal (Fig. 3Q–T) tier.

In the dorsal region, the PgvUV and Pgb labeling was detected in the cell bodies of R1 or R2 photoreceptors in a complementary manner, revealing three types of ommatidia (Fig. 3E,F): type I-d (solid circles), which expresses each mRNA in one of these cells; type II-d (dotted circles), which expresses PgvUV in both cells; and type III-d (broken circles), which expresses Pgb in both cells. The R3–R8 photoreceptors were all labeled with the Pgl2 probe (Fig. 3G,K). Labeling with the Pgl3 probe was undetectable in the dorsal region (Fig. 3H,L; see also Fig. 3D). Among 398 dorsal ommatidia, we found significantly more of type I-d (199 ommatidia, 50%), than of type II-d (108, 27%) or type III-d (91, 23%) (chi-square test,  $\chi^2=12.76$ , d.f.=2).

The labeling pattern was more complicated in the ventral region. The PgvUV labeling was of two types: one containing either one of R1 or R2 labeled with the probe, whereas the other had R1 and R2 both labeled (Fig. 3M). In the ventral type I (type I-v), the unlabeled R1 or R2 was recognized by the Pgb probe and R3–R8 were recognized by the Pgl2 probe, but not by the Pgl3 probe, as in type I-d in the dorsal region (solid circles in Fig. 3M,N). The other type was particularly special: all the R1 and R2 were also strongly labeled with the Pgb probe. These cells thus co-express two opsins from different clades. This type can be divided further according

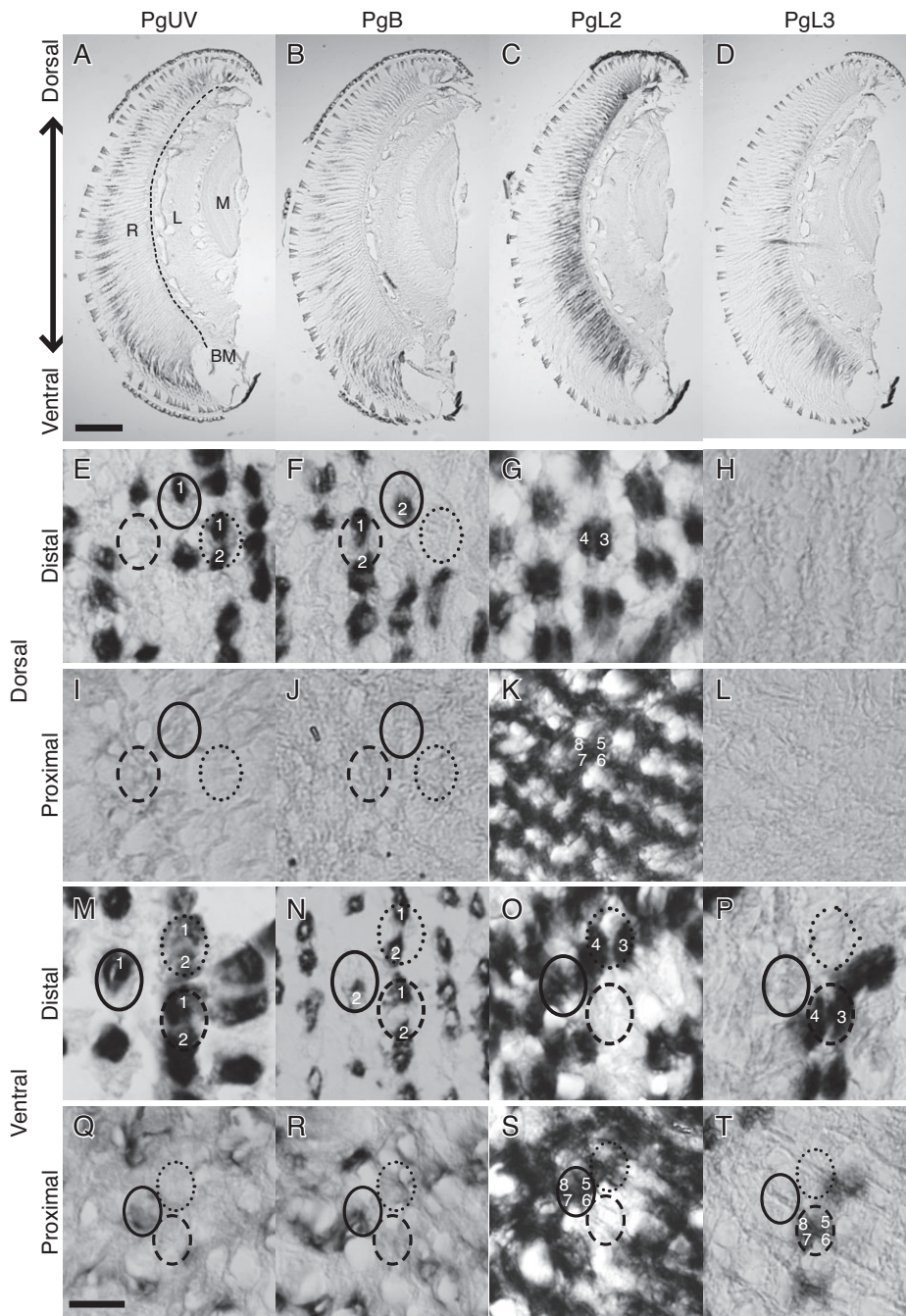


Fig. 3. Localization of four opsin mRNAs by *in situ* hybridization (A–D, longitudinal sections; E–L, transverse sections of the dorsal region; M–T, transverse sections of the ventral region). The sections were labeled with probes specific to the PgUV (A,E,I,M,Q), PgB (B,F,J,N,R), PgL2 (C,G,K,O,S) or PgL3 (D,H,L,P,T) mRNAs. Two sets of serial sections through the distal (E–H and M–P) and proximal tier (I–L,Q–T) showed the existence of three types of ommatidia in both the dorsal and the ventral regions. The ommatidial types were indicated by solid (type I), dotted (type II) and broken (type III) circles in both regions. Scale bars: in A, 200  $\mu$ m for A–D; in Q, 20  $\mu$ m for E–T. Numbers indicate photoreceptors R1–R8, BM, basement membrane; L, lamina; M, medulla; R, retina.

to the labeling with the PgL2 and PgL3 probe: one type has R3–R8 labeled with the PgL2 probe (dotted circles in Fig. 3O,S, type II-v), whereas the other has these cells labeled with the PgL3 probe (broken circles in Fig. 3P,T, type III-v). Among 311 ventral ommatidia, we found significantly more of the type I-v (156 ommatidia, 50%), than of the type II-v (56, 18%) or the type III-v (99, 32%) ( $\chi^2=15.31$ , d.f.=2). There was no significant difference in the fractions between the dorsal and ventral regions ( $\chi^2=3.48$ , d.f.=2).

Taken together, we identified six types of ommatidia, as summarized in Fig. 4. Type I-d and type I-v are identical in terms of the opsin expression pattern, but are different in the size of the R1 and R2 cell body (see next section). The type II-d, type III-d, type II-v and type III-v are each found in different fractions.

#### Anatomical heterogeneity of ommatidia

We investigated the general anatomical features of the ommatidia in unstained plastic sections, and thus identified three types of ommatidia in the ventral region (Fig. 5). The ventral ommatidia were characterized firstly by the size of the cell bodies of the R1 and R2 photoreceptors. In about half of the ommatidia, either one of R1 or R2 was larger than the other (Fig. 5A). These ‘asymmetric’ ommatidia most likely correspond to type I identified by *in situ* hybridization, which occupies about half of the ommatidia (Fig. 4). The labeling with the PgUV and PgB probes (Fig. 3M,N) suggests that the larger cells express the PgUV mRNA. The other ommatidia have R1 and R2 cell bodies of similar size (Fig. 5B,C), and are further divided into two types: with and without red pigment (arrowheads in Fig. 5C) around the rhabdom. Such pigment functions as a red

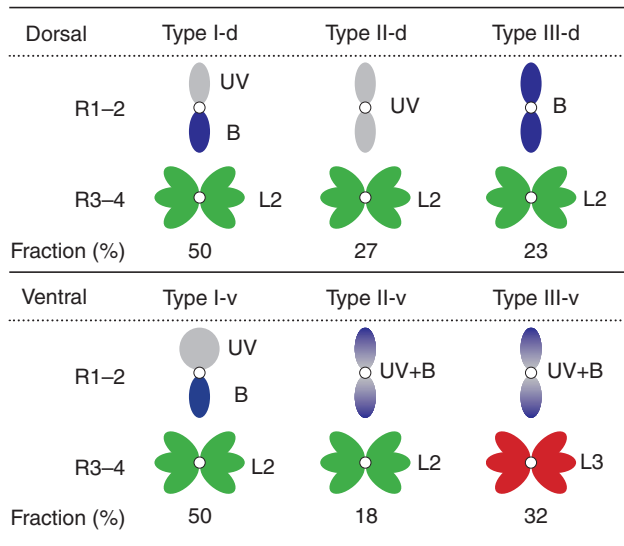


Fig. 4. Six types of ommatidia in the eye of *Parnassius glacialis* with their fractions. The localization of PgUV and PgB in type I-d and type I-v ommatidia might be changed. PgUV and PgB are co-expressed in the R1 and R2 photoreceptors of type II-v and III-v ommatidia. The opsins of the small R9 photoreceptors could not yet be determined.

spectral filter; in *Papilio xuthus*, the filtering effect of pigment together produces red receptors peaking at 600 nm with PxL3 and a visual pigment with peak wavelength at about 575 nm (Arikawa et al., 1999). Therefore, it is most likely that the red pigmented ommatidia of *Parnassius glacialis* are of the ventral type III expressing PgL3 (Fig. 4), the ortholog of PxL3, in R3–R8 photoreceptors. The non-pigmented ommatidia presumably correspond to the ventral type II. Among 137 ommatidia, we found significantly more of the type I (61 ommatidia, 45%), than the type II (32, 23%) or the type III (44, 32%) ( $\chi^2=7.34$ , d.f.=2). The fraction values were not significantly different from the values identified by *in situ* hybridization ( $\chi^2=0.88$ , d.f.=2).

## DISCUSSION

### Molecular evolution of L opsins in Papilionidae

We found two L opsins in the glacial Apollo butterfly, *Parnassius glacialis*. The other two papilionid species studied so far, *Papilio xuthus* and *Papilio glaucus*, have at least three L opsins (L1–L3) (Kitamoto et al., 1998; Briscoe, 2000), but *Parnassius glacialis* has no L1 ortholog. Among these L opsins, the green-absorbing L2 is probably the ancestral form because of two reasons. First, in species with only one L opsin (e.g. *Manduca*, *Vanessa*, *Pieris*; see Fig. 1C), the L opsins are all the green-absorbing type, expressed in the R3–R8 photoreceptors of all ommatidia. Second, in both *Papilio* (Kitamoto et al., 1998; Arikawa, 2003; Briscoe, 2008) and *Parnassius* (Figs 3, 4), the L2 orthologs are expressed in a majority of photoreceptors throughout the eye.

The first duplication of the ancestral L opsin gene happened before the subfamily Parnassiinae diverged from the other papilionid subfamilies, probably in the Cretaceous period (Grimaldi and Engel, 2005; Omoto et al., 2009), giving rise to the L2 and L3 opsins. The peak absorption of L3 opsin was shifted to a longer wavelength. In *Papilio*, L3 opsin is expressed in a subset of receptors that also contain a red perirhabdomal filter pigment (Arikawa, 2003). The sensitivity profile of these receptors is rather narrow peaking at

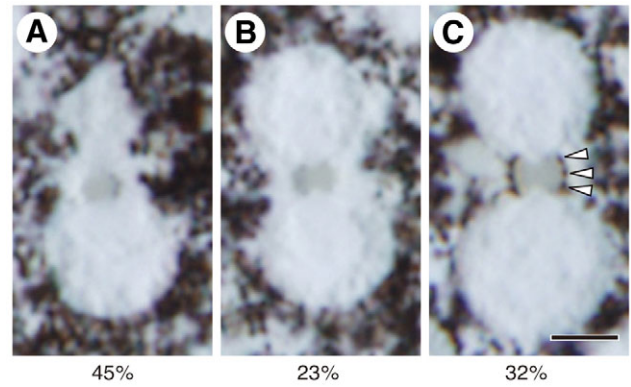


Fig. 5. Unstained transverse sections of plastic-embedded retina in the ventral region. (A–C) Three types of ommatidia were identified, corresponding to the three types identified by *in situ* hybridization (see Fig. 3). Arrowheads in C indicate the red filter pigment surrounding the rhabdom. Ratios of the three types in the region are shown below the pictures.

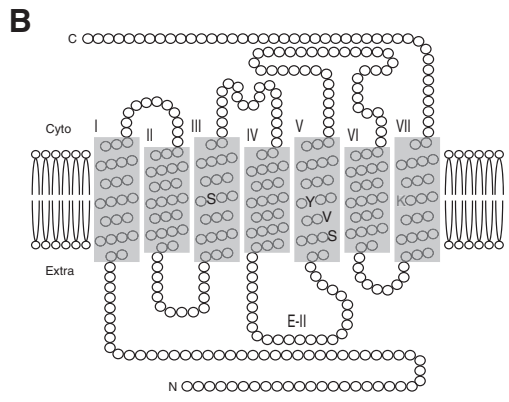
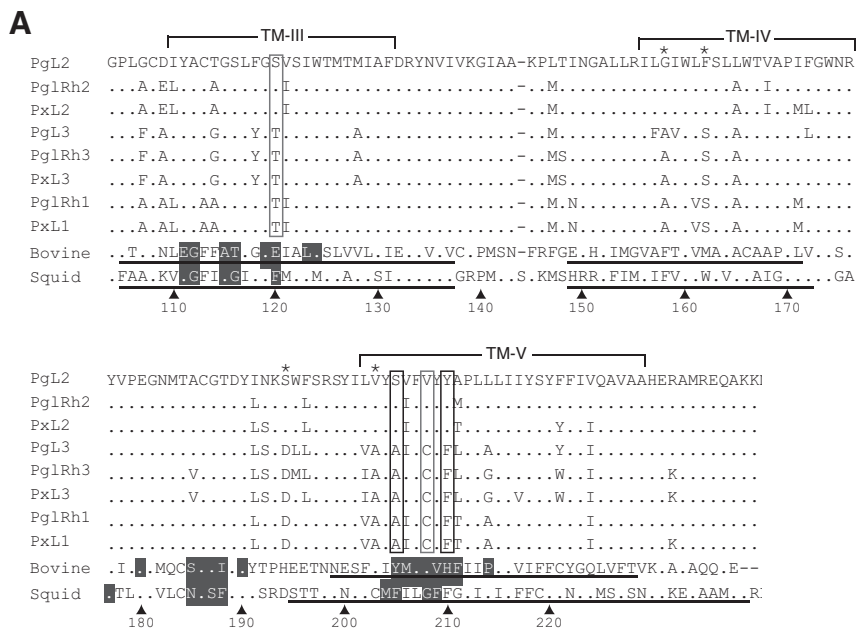
600 nm, which is well reproduced by postulating an orange-absorbing visual pigment ( $\lambda_{\max}=575$  nm, PxL3) and a filtering effect of the red perirhabdomal pigment. Moreover in *Papilio*, PxL3 is co-expressed with PxL2 in another subset of photoreceptors. These photoreceptors have acquired a broad-band sensitivity owing to the green-absorbing visual pigment ( $\lambda_{\max}=515$  nm, PxL2) and the 575 nm-absorbing PxL3. The PxL3 further duplicated in the lineage of *Papilio*, producing PxL1, which is however minor and expressed always with PxL2, in a limited number of photoreceptors only in the ventral region (Kitamoto et al., 1998).

### Spectral tuning of L opsins

The four *Parnassius* opsins all have seven transmembrane domains (TM-I–TM-VII) with a lysine residue in TM-VII for binding the chromophore. To predict the amino acid residues involved in the evolution of the orange-absorbing L3 from the green-absorbing L2, we compared the amino acid sequences of papilionid L opsins with those of bovine and squid rhodopsins whose 3D structures have been determined (Fig. 6). In the region from TM-III to TM-V that forms the chromophore-binding pocket (Murakami and Kouyama, 2008), we first listed the amino acid residues located within 5 Å from any carbon of the chromophore in either the bovine or squid rhodopsins (Fig. 6A). We thus identified 24 sites in TM-III and TM-V, and the extracellular loop II connecting TM-IV and TM-V (E-II in Fig. 6B). We then compared the corresponding amino acids in the papilionid L opsins: 16 out of the 24 residues were conserved in all papilionid L opsins, indicating that they serve the basic function of L opsins.

Among the remaining eight residues, four (120 in TM-III; 205, 208, 210 in TM-V; numbering according to the squid rhodopsin, boxed in Fig. 6A) have different amino acids between the L2 group and the duplicated L1/L3 group. At site 205, the L2 group has a serine whereas the L1/L3 group has an alanine (Ser205Ala), and at site 210 the tyrosine in L2 is replaced by phenylalanine in L1/L3 (Tyr210Phe). Both of these substitutions remove the OH group from the L2 opsins. Other sites are Ser120Thr and Val208Cys.

Accumulated evidence has suggested that introduction and removal of OH groups around the chromophore are crucial in spectral tuning both in vertebrates (Merbs and Nathans, 1993) and invertebrates (Frentiu et al., 2007). Frentiu et al. (Frentiu et al., 2007)



**C**

| Site   | 120 | 205 | 208 | 210 | 305 |
|--------|-----|-----|-----|-----|-----|
| L2     | Ser | Ser | Val | Tyr | Lys |
| L1/L3  | Thr | Ala | Cys | Phe | Lys |
| Bovine | Glu | Tyr | Val | His | Lys |
| Squid  | Phe | Phe | Gly | Phe | Lys |

Fig. 6. (A) Alignment of partial sequences of Papilionidae L opsins with the bovine and squid rhodopsins. TM-III, TM-IV and TM-V indicate the transmembrane domains III, IV and V of Papilionid opsins, respectively. The TM domains of the bovine and squid rhodopsins are underlined in each sequence. Shaded residues in the bovine and squid rhodopsins are localized within 5 Å from any carbon of the chromophore. The four boxed sites are potential candidate amino acids responsible for spectral tuning. Numbering is based on the squid opsin. (B) Diagram of L2, indicating the four boxed sites shown in A and the lysine (K) in TM-VII required for chromophore attachment. (C) Amino acids at the four sites in L2, L1/L3, bovine and squid opsins, as well as the lysine for chromophore attachment.

showed that in the L opsins of nymphalid butterflies of the genus *Limnitis*, the Ser–Ala substitution at 163 (according to our numbering) in TM-IV is associated with a short wavelength shift from 530 to 514 nm. However, at site 90 in the *Limnitis* L opsins, the same Ser–Ala substitution is associated with a long wavelength shift, indicating that the mechanism is not simple. In the small white butterfly, *Pieris rapae*, the Tyr–Phe substitution in E-II causes a long wavelength shift in their B opsins (Wakakuwa et al., in press).

Most probably, the two residues at the sites 205 and 210 (boxed in Fig. 6A) play a crucial role in the spectral tuning of L2 and L1/L3. A further understanding of the contribution of the other two residues (also boxed; and of other residues, e.g. those indicated by asterisks in Fig. 6A) must await spectroscopic analyses of opsin molecules with point mutations.

**Eye regionalization and characteristic expression pattern**

We found a clear dorsoventral specialization in the eye of *Parnassius glacialis*. Firstly, the dorsal ommatidia are free from pigmentation around the rhabdom, which has also been found in other butterflies (Ribi, 1979; Arikawa et al., 2009). Secondly, the expression pattern of opsin in the dorsal ommatidia is rather simple, similar to other insects that have only one L opsin (Briscoe et al., 2003; White et al., 2003; Wakakuwa et al., 2004; Arikawa et al., 2005; Spaethe and Briscoe, 2005; Wakakuwa et al., 2005). The L opsin expressed in the dorsal region of the *Parnassius* eye is PgL2. The simple and

basic organization of the dorsal ommatidia of *Parnassius glacialis* with ancestral L opsin suggests that the dorsal region retains its ancestral nature, whereas the ventral region has been reorganized for specific visual functions, such as colour vision.

There are three types of ommatidia in both the dorsal and ventral regions. Throughout the eye, one half of the ommatidia are of the type I, containing one UV and one B receptor, which is common in butterflies (e.g. Arikawa and Stavenga, 1997; Awata et al., 2009). Another feature common among insects is that the ventral region is more sensitive to longer wavelengths, which is also the case here. In the ventral region, the type III-v ommatidia have red pigment around the rhabdom (Fig. 5). The R3–R8 photoreceptors of these ommatidia express PgL3 mRNA, the ortholog of the *Papilio* orange opsin PxL3 (Fig. 4). In *Papilio*, the 575 nm-absorbing PxL3 produces 600 nm-peaking red receptors with the aid of the filtering effect of a red perirhabdomal pigment (Arikawa et al., 1999). The combination of PgL3 and red pigment in *Parnassius glacialis* most probably produces red receptors, at least in the proximal tier, where the effect of the red filter is pronounced.

Another unique feature of the *Parnassius* eye is the molecular heterogeneity of the distal R3 and R4 photoreceptors. Assuming that PgL2 is green absorbing and PgL3 is orange absorbing, the spectral sensitivities of R3 and R4 are variable depending on the ommatidial types (Fig. 4). In *Papilio xuthus*, R3 and R4 are all green sensitive, providing a single spectral input system arranged in the

complete hexagonal lattice and having a high spatial and time resolution (Takemura et al., 2005; Takemura and Arikawa, 2006). This function might not be necessary for the Apollo, a member of a slow-flying and ancestral group of papilionids.

It should also be noted that the eye of *Parnassius glacialis* has a subset of R1 and R2 co-expressing PgUV and PgB (Fig. 4). Co-expression of multiple opsins now appears to be more common than previously thought, but the case of *Parnassius glacialis* is the first example of a combination of UV and B opsins. Reported combinations were rather diverse: two L types in *Papilio* (Arikawa et al., 2003), two B types in a pierid species, *Colias erate* (Awata et al., 2009), a B type and an L type in a lycaenid species, *Lycaena rubidus* (Sison-Mangus et al., 2006), and two UV types in the fruitfly *Drosophila melanogaster* (Mazzoni et al., 2008; Stavenga and Arikawa, 2008). The photoreceptors expressing both the PgUV and PgB visual pigments are assumed to have a rather broad spectral sensitivity in the short wavelength region, and our preliminary electrophysiological measurement in *Parnassius glacialis* has indicated a similar spectrally extended sensitivity (data not shown).

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