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ORIGINAL ARTICLE

Ubiquitous distribution of helmchrome in phototactic swarmers of the stramenopiles

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Abstract Most swarmers (swimming cells) of the stramenopile group, ranging from unicellular protist to giant kelps (brown algae), have two heterogeneous flagella: a long anterior flagellum (AF) and a relatively shorter posterior flagellum (PF). These flagellated cells often exhibit phototaxis upon light stimulation, although the mechanism by which how the phototactic response is regulated remains largely unknown. A flavoprotein concentrating at the paraflagellar body (PFB) on the basal part of the PF, which can emit green autofluorescence under blue light irradiance, has been proposed as a possible blue light photoreceptor for brown algal phototaxis although the nature of the flavoprotein still remains elusive. Recently, we identified helmchrome as a PF-specific flavoprotein protein in a LC-MS/MS-based proteomics study of brown algal flagella (Fu et al. 2014). To verify the conservation of helmchrome, in the present study, the absence or presence and the localization of helmchrome in swarmers of various algal species were investigated. The results showed that helmchrome was only detected in phototactic swarmers but not the non-phototactic ones of the stramenopile group. Electron microscopy further revealed that the helmchrome detectable swarmers bear a conserved PFB-eyespot complex, which may serve as structural basis for light sensing. It is speculated that all three conserved properties: helmchrome, the PFB structure, and the eyespot apparatus, will be essential parts for phototaxis of stramenopile swarmers.

Keywords Brown algae · Flagella · Helmchrome · Photoreceptor · Phototaxis · Stramenopiles

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Abbreviations

AUREO	Aureochrome
bZIP	Basic leucine zipper
ChR	Channelrhodopsin
DAPI	4′,6-Diamidino-2-phenylindole
HELMC	Helmchrome
LOV	Light-oxygen-voltage sensing
PAC	Photoactivated adenylyl cyclase
PAS	Per-ARNT-Sim
PFB	Paraflagellar body
PHOT	Phototropin
PHY	Phytochrome
RGS	Regulator of G protein signaling
VVD	Vivid
WC	White collar
ZnF	Zinc finger

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Introduction

Blue light (BL) is one of the most vital environmental factors that regulate various biological activities, and for sensing the BL, organisms of algae, fungi, and land plants have evolved diverse proteins functioning as BL photoreceptors. Many of these proteins bear one or more light, oxygen, and voltage sensing (LOV) domain serving as light sensing motif. The LOV domain is a subset of Per-ARNT-Sim (PAS) superfamily that binds flavin as chromophore in photoreceptors from land plants to the stramenopiles (Crosson et al. 2003; Krauss et al. 2009; Losi and Gärtner 2012). The most well-studied LOV domain-containing BL photoreceptor is phototropin (PHOT), which was identified in *Arabidopsis* (Huala et al. 1997) and widely conserved in green algae and land plants with multiple functions in mediating phototropism, stomatal opening, or chloroplast moving and so on (see reviews in Christie 2007; Kami et al. 2010; Kianianmomeni and Hallmann 2014). Land plants also possess another LOV protein family, ZTL/FKF1/LKP2, which can mediate circadian rhythmicity and flowering (Ito et al. 2012). In circadian system of fungi *Neurospora crassa*, three PAS or LOV domain-containing proteins, white collar-1 (WC-1) (Ballario et al. 1996), white collar-1 (WC-2) (Crosthwaite et al. 1997; Cheng et al. 2003a, b), and vivid (VVD) (Heintzen et al. 2001), play essential roles in regulating BL responses. In the stramenopiles, aureochrome (AUREO) is the major LOV domain-containing BL receptor, which was identified as a transcriptional factor in regulating photomorphogenesis, e.g., BL-induced branching (Takahashi et al. 2007). AUREO is localized at nucleus (Takahashi et al. 2007) and widely distributed in photosynthetic stramenopiles (Ishikawa et al. 2009; Cock et al. 2010; Deng et al. 2014).

Different LOV domain-containing BL photoreceptors often possess diverse regulating modules in addition to LOV domain(s) and/or other PAS domain(s) (Fig. 1). For example, PHOT has two LOV domains (PHOT_LOV1 and PHOT_LOV2) coupled to a serine/threonine protein kinase; AUREO has one basic leucine zipper (bZIP) domain linked to the N terminus region of one LOV domain (AUREO_LOV); phytochrome 3 (PHY3) (also known as neochrome), a chimeric protein identified in fern *Adiantum* (Nozue et al. 1998; Suetsugu et al. 2005), has a combination of a phytochrome and a full PHOT domain. The fugal WC-1 and WC-2 both bear zinc-finger DNA-binding domains, while the small VVD protein has only one LOV domain (VVD_LOV) that can partially replace the function of the WC1_LOV domain (Cheng et al. 2003a, b).

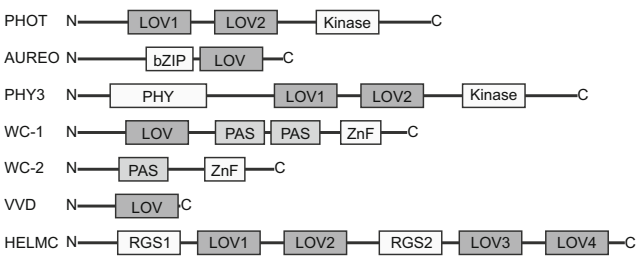

Fig. 1 Schematic diagram of the domain structures of helmchrome and six LOV/PAS domain-containing BL photoreceptors. *PHOT* phototropin; *AUREO*, aureochrome; *PHY3*, phytochrome 3; *WC-1*, white collar-1; *WC-2*, white collar-2; *VVD*, vivid; *HELMC*, helmchrome; *LOV*, light, oxygen, and voltage sensing; *bZIP*, basic leucine zipper; *PHY*, phytochrome; *PAS*, Per-ARNT-Sim; *ZnF*, zinc finger; *RGS*, regulator of G protein signaling

Most of the unicellular algal cells, including reproductive cells of multicellular algae (i.e., zoospores and gametes), are capable of sensing incident light orientation and subsequently swimming toward (positive phototaxis) or away from (negative phototaxis) the light source. Algae have evolved diverse proteins and light signaling pathways for photoinduced orientation, and two distinct photoreceptors have been identified in the unicellular algal groups (Hegemann et al. 2001; Hegemann 2008; Kianianmomeni and Hallmann 2014). In the green alga *Chlamydomonas*, phototaxis is mainly mediated by channelrhodopsins (ChR1 and ChR2) (Nagel et al. 2002, 2003; Sineshchekov et al. 2002), which are localized at the eyespot apparatus and binds all-trans retinal as chromophore (Foster et al. 1984; Suzuki et al. 2003). While at the base of the long flagellum of *Euglena gracilis* Klebs, a paraflagellar swelling that can emit green autofluorescence when stimulated by blue light (365 to 436 nm) has been considered as the ciliary photoreceptor for decades (Kivic and Vesk 1972; Barsanti et al. 1997). Iseki et al. (2002) finally identified two homologous photoactivated adenylyl cyclase (PAC) as the blue light receptors responsible for phototaxis in this species, and the authors confirmed that the photoreceptor localized at the paraflagellar swelling and bind FAD as the chromophore. In addition to the algal phototactic swarmers, flagellated swimming zoospores of several fungus species also show phototaxis (Saranak and Foster 1997), and a recent study revealed a novel rhodopsin-guanylyl cyclase protein involved in the phototactic signaling pathway (Avelar et al. 2014).

The phototactic behavior of brown algae swarmers (Phaeophyceae) has been intensively investigated. Kawai et al. (1990, 1991) reported that gametes of *Ectocarpus siliculosus* (Dillwyn) Lyngbye and zoospores of *Pseudochorda gracilis* Kawai & Nabata were sensitive to the light wavelengths between 370 and 520 nm with two major peaks at 420–430 and 450–460 nm. These results suggest that the phototactic responses of brown algae swarmers are mediated by blue light. A strong negative phototaxis was also reported in swarmers of *Scytosiphon lomentaria* (Lyngbye) Link and *Petalonia fascia* (O. F. Müller) Kuntze under photon irradiances of 10–90 μmol m^{-2} s^{-1} (Flores-Moya et al. 2002). It has been speculated that the ability of these swarmers to sense light is anatomically relied on their flagella. Brown

algae belong to the Stramenopiles, and the swarmers are characterized by bearing two heterogeneous flagella, which are laterally inserted into the cell body. The long anterior flagellum (AF) is decorated with tubular mastigonemes and generates forces through undulating bending to propel the cell swim forward, while the shorter posterior flagellum (PF) lacks mastigonemes, extends backward, and often possesses a basal swelling structure designated paraflagellar body (PFB, Moestrup 1982). The PF of phototactic swarmers emits green autofluorescence (515–520 nm) under blue blight excitation (440 nm), which indicates that flavin might be the chromophore bind to the blue light photoreceptor localizing at the PF (Müller et al. 1987; Coleman 1988; Kawai 1988, 1992). In vivo, the density of the green autofluorescence increases as the PF elongates at the early developmental stage of brown algal swarmers (Fu et al. 2013). In addition, the PFB and eyespot apparatus are only present in swarmers of phototactic brown algal species and are absent in non-phototactic ones (Müller et al. 1987; Kawai 1988, 1992). The presence or absence of the green autofluorescence of the flagella correlates with that of the PFB and eyespot apparatus. Moreover, a high-speed video microscopic observation demonstrated that rapid lateral beating of the PF changed the swimming orientation of swarmers in phototactic responses (Matsunaga et al. 2010). These studies support the hypothesis that the flavoprotein specifically localized at the PF has a blue light receptor function for phototaxis of brown algal swarmers.

In our recent proteomics analysis of brown algal flagella, we identified a PF-specific protein designated "helmchrome," which was shown to be one of the most abundant proteins in the PF proteome database (Fu et al. 2014). Helmchrome (HELMC) has a predicted molecular weight of 167 kDa and contains two Regulator of G protein Signaling (RGS) domains and four LOV domains. It is predicted to be a flavoprotein because the 11 amino acid residues responsible for FMN binding (Crosson and Moffat 2001) are highly conserved in its LOV1 and LOV3 domains (Fu et al. 2014). We raised a polyclonal antibody against helmchrome, and using this affinity-purified antibody, we confirmed that helmchrome was specifically localized along the entire length of the PF with significant enrichment at the PFB in brown algal swarmers of *Colpomenia bullosa* (D.A. Saunders) Yamada. Immunoelectron microscopy showed that helmchrome proteins were particularly localized at the crystalized material zone of the PFB, indicating that they were directly facing the eyespot structure. Because many algal swarmers, particularly for those belonging to the stramenopiles, share similar flagellar characteristics such as green autofluorescence under BL irradiance and the PFB structure, it is questioned whether helmchrome is conserved in these cells. In order to answer this question, we conducted investigations using the anti-helmchrome antibody to examine the presence or absence of helmchrome in the swarmers of various algal species. Electron microscopy was also used to compare the ultrastructural features of flagella in several swarmers. The results confirmed that helmchrome is only conserved in the phototactic swarmers of the stramenopiles, indicating that it might play an important role in phototaxis.

Materials and methods

Phylogenetic analysis

The amino acid sequences of phototropin, aureochrome, phytochrome 3 (neochrome), white collar-1, white collar-2, and vivid proteins were downloaded from NCBI (www.ncbi.nlm.nih.gov). The LOV or PAS domains were predicted with Pfam database (Finn et al. 2014). Information including the accession number for each protein, searching results for each LOV/PAS domain in Pfam database was summarized in mentary Table S1. Maximum likelihood tree was constructed with MEGA6 (Tamura et al. 2013) based on the LG model. Bootstrap analysis was calculated for 1000 replications. Multiple sequence alignments of LOV domains were done with Clustal X 2.0 (Larkin et al. 2007).

Algae collection and culture

The brown and green algae

Mature thalli of *C. bullosa*, *Analipus japonicas* (Harvey) M. J. Wynne, *S. lomentaria* (Lyngbye) Link, *Leathesia difformis* Areschoug, *Melanosiphon intestinalis* (De A. Saunders) M. J. Wynne, *Desmarestia ligulata* (Stackhouse) J. V. Lamouroux, *Alaria crassifolia* Kjellman, *Saccharina angustata* (Kjellman) C. E. Lane, C. Mayes, Druehl & G. W. Saunders, *Saccharina japonica* (Areschoug) C. E. Lane, C. Mayes, Druehl & G. W. Saunders, *Undaria pinnatifida* (Harvey) Suringar, *Agarum clathratum* Dumortier, *Ulva pertusa* Kjellman, and *Bryopsis plumosa* (Hudson) C. Agardh were collected from the field from February to October in 2013 and 2014 along the coastline in Muroran, Hokkaido, Japan. *Chorda filum* (Linnaeus) Stackhouse was collected in Oshoro, Hokkaido, Japan in June 2014. The freshly collected samples were washed several times with autoclaved seawater, wiped with paper towels, put into petri dishes, and placed in darkness at 10 °C overnight. Swarmers were released by pouring chilled seawater onto the thalli the next day. Sperms of *Fucus distichus* Linnaeus, *Silvetia babingtonii* Harvey, and *Sargassum confusum* C. Agardh were obtained by directly excising the receptacles from algal thalli and exerting pressure on the antheridia with a glass slide (Motomura 1994; Nagasato et al. 2001). Maturation of male and female gametophytes of *S. japonica* was induced by transferring Fe-free $ASP_{12}NTA$ medium to half-strength PES

medium (Provasoli 1968) and cultured under long-day conditions (20–40 μmol m^{-2} s^{-1}, 14 h light:10 h dark) at 10 °C (Motomura and Sakai 1981). Release of sperm was induced by adding the culture medium from the mature female gametophyte to the mature male gametophytes (Müller et al. 1979).

The Labyrinthulomycetes

Sicyoidochytrium sp., *Ulkenia amoeboidea* (Bahnweg & Sparrow) Gaertner, *Ulkenia* sp., *Parietichytrium sarkarianum* (Gaertner) Yokoyama, Shalleh & Honda, and *Parietichytrium* sp. were cultured on dGPY medium (agarose 1 %, glucose 0.2 %, peptone 0.1 %, and yeast extract 0.05 % in seawater) and *Thraustochytrid-like* 12B was cultured on a modified F medium (agarose 1 %, glucose 8 %, peptone 1 %, and yeast extract 1 % in 50 % seawater) (Perveen et al. 2006) under long-day conditions (20–40 μmol m^{-2} s^{-1}, 14 h light:10 h dark) at 20 °C. Release of zoospores of these Labyrinthulomycetes was induced by transferring the cell pellet from agar to seawater and incubating at room temperature for about 3 h.

The crysophycean, xantophycean, and haptophycean algae

Dinobryon sociale (Ehrenberg) Ehrenberg, *Dinobryon sertularia* Ehrenberg, and *Ophiocytium majus* Nägeli were cultured in DY-V medium at 15 °C under long-day conditions (20–40 μmol m^{-2} s^{-1}, 16 h light:8 h dark). *Ochromonas danica* Nägeli was cultured in a medium containing glucose 0.1 %, tryptone 0.1 %, yeast extract 0.1 %, and liver extract 4 % at 20 °C under long-day conditions (20–40 μmol m^{-2} s^{-1}, 16 h light:8 h dark). *Synura petersenii* Korshikov and *Chrysochromulina hirta* Manton from the Microbial Culture Collection at the National Institute for Environmental Studies (http://mcc.nies.go.jp) were cultured in AF-6 and ESM medium (see http://mcc.nies.go.jp/02medium.html), respectively, at 15 °C under long-day conditions.

The dinoflagellate algae

Symbiodinium sp., *Symbiodinium* sp., *Bysmatrum* sp., and TM106 *Gymnodinium* sp. (all deposited in Faculty of Science, Hokkaido University) were cultured in half strength of PES medium at 15 °C under long-day conditions.

Immunofluorescence microscopy

Swimming swarmers were fixed for 30 min at room temperature with 0.1 % glutaraldehyde and 3 % paraformaldehyde in PIPES 60 mM, HEPES 25 mM, EGTA 10 mM, and $MgCl_2$ 2 mM (PHEM) buffer. For marine species, 2 % $NaCl_2$ was added to the fixative. Samples were washed three times with PBS and then attached to poly-L-lysine coated (0.1 mg/ml) cover glasses. After treatments with 5 % Trion X-100 in PBS for 30 min and 0.1 % $NaBH_4$ in PBS for 20 min, samples were treated with blocking solution (2.5 % skim milk, 5 % normal goat serum, 0.1 % NaN_3 in PBS) for 30 min at 37 °C. The primary antibody mixture was composed of monoclonal anti-α-tubulin antibody (DM1A, Sigma-Aldrich) and polyclonal anti-helmchrome antibody, which were diluted 200 and 800 times in PBS, respectively. The anti-helmchrome antibody was generated in previous work (Fu et al. 2014). Samples were incubated with the mixture at 20 °C overnight, washed three times with PBS, and then treated with the secondary antibody mixture (FITC-conjugated goat anti-rabbit IgG and TRITC-conjugated goat anti-mouse IgG, Sigma-Aldrich; both diluted 50 times in PBS) for 60 min at 37 °C. Afterwards, they were stained with 4′,6-diamido-2-phenylindole (DAPI, 0.5 μg/ml in PBS) for 10 min at room temperature and finally mounted in Mowiol 4–88 mounting medium (Osborn and Weber 1982) containing 0.2 % *p*-phenylenediamine. Observations were performed with an epifluorescence microscope (BX50-FLI, Olympus, Tokyo, Japan). Images were captured using a digital camera (AxioCam MRm and Axio-Vision systems, Carl Zeiss, Germany).

Autofluorescence of flagella in living materials was observed under BV excitation (400–440 nm) using an epifluorescence microscope (BX50-FLI, Olympus, Tokyo, Japan).

Phototaxis examination

Flagellate cells including Chrysophyceae, Xanthophyceae, and Labyrinthulomycete species were inoculated (for species collected from the field) in petri dishes with unilateral illumination (fluorescent white light, 30–40 μmol photons m^{-2} s^{-1}), and the phototactic responses were determined. Similarly, phototaxis of the swarmers of the brown, green, and schizocladiophycean algae was examined; plurispores of *E. siliculosus* and *C. bullosa*, zoospores of *A. japonicas*, male and female gametes of *S. lomentaria*, zoospores of *M. intestinalis*, plurispores of *L. difformis*, female gamete of *Culteria cylindrica*, zoospores and sperms of *D. ligulata*, zoospores of *C. filum*, zoospores of *S. angustata*, sperms and zoospores of *S. japonica*, zoospores of *A. clathratum*, zoospores of *A. crassifolia*, zoospores of *U. pinnatifida*, zoospores of *Schizocladia ischiensis*, male and female gametes of *U. pertusa*, female gamete of *B. plumosa*.

Phototaxis of sperms of *F. distichus* was referred to a previously study (Müller et al. 1987). Phototaxis of sperms of *S. babingtonii* and *S. confusum*, zoospores of *Ophiocytium maius*, *S. petersenii*, *C. hirta*, and *Gymnodinium* sp. was not determined in the present study, nor relevant reference was available.

Transmission electron microscopy

Samples were prepared by rapid freezing and substitution techniques as described in Nagasato and Motomura (2002). Briefly, swarmers were collected by centrifugation (3500 rpm for 1 min), and the obtained pellets were mounted on formvar-coated gold loops (5–10 mm in diameter). The cells were rapidly frozen by plunging the loops into liquid propane, which had been precooled to −180 °C, and then, the loops were transferred into liquid nitrogen and finally stored in fixative (2 % OsO_4 in acetone) at −85 °C for 2 days. After 2 days, the samples were held at −20 °C for 2 h, 4 °C for 2 h, and finally allowed to warm to room temperature. After several washes with acetone, the samples were gradually infiltrated with Spurr's epoxy resin (Spurr 1969) at room temperature and finally embedded in the resin. Serial sections of the samples were cut with a diamond knife (Diatome, Hatfield, PA, USA), mounted on formvar-coated slot grids and stained with 4 % uranyl acetate or 50 % Ti blue (Nisshin EM, Tokyo, Japan) and Reynolds' lead citrate (Reynolds 1963) at room temperature. Observations were carried out with a JEM-1011 electron microscope (JEOL, Tokyo, Japan). Sperm of *S. confusum* was prepared by chemical fixation. Excised conceptacles from receptacles were fixed for 1 h on ice with 2 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 2 % NaCl and 0.1 % $CaCl_2$. The samples were washed for several times with the 0.1 M cacodylate buffer and then post-fixed with 2 % OsO_4 in the same buffer on ice for 1 h. Afterwards, they were dehydrated in an acetone series and embedded in Spurr's epoxy resin.

Results and discussion

Phylogenic analysis of the LOV domains of helmchrome

Since the conservation and critical functions of LOV domains, in order to analyze the phylogenetic relationship of HELMC_LOV domains with that of known BL photoreceptors, a maximum likelihood (ML) tree (Fig. 2a) for LOV and PAS domains was constructed with 73 sequences (Supplementary Table S1). The phylogenetic tree shows that HELMC_LOVs form two clades referred to HELMC_LOV1, 3 and HELMC_LOV2, 4, which are diverged from other clades including PHOT_LOV1, PHOT_LOV2, AUREO_LOV, WC1_LOV, and WC_PAS. The cysteine, which is essential for binding chromophore (Crosson and Moffat 2001), is replaced by a serine residue in HELMC_LOV2, 4 (Fig. 2b). The following three residues, Arg-Gln-Ile and Lys-Gln-Val, respectively, share similar characters between the two groups. However, the subsequent arginine residue is not conserved in HELMC_LOV2, 4. These differences indicate that the two types of HELMC_LOV

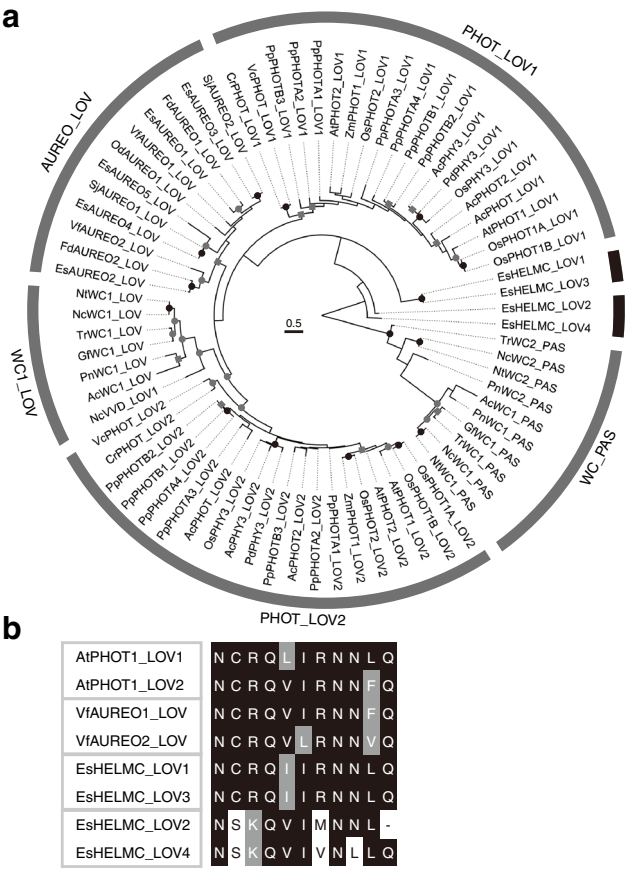

Fig. 2 Phylogenetic analysis of LOV/PAS domains from selected BL photoreceptors. **a** A maximum likelihood tree constructed from 73 LOV/PAS sequences. Five clades of known BL photoreceptors are labeled with *gray bars*, and LOV domains of helmchrome are indicated in *black color*. Brach lengths are constructed to scale, and the scale bar represents the number of amino acid substitutions per site. Supporting values are indicated before branch nodes as *black dots* (over than 90 %), *gray dots* (70~90 %), and *gray squares* (50~70 %). The values less than 50 % are not shown. Information for each protein/sequence is summarized in supplementary Table S1. **b** Multiple alignments of the11 amino acid residues essential for binding FMN. Same residues are indicated by *black backgrounds*. Residue changing into a similar one is indicated by *gray background*. The *dashes* indicate the unknown residues

domains might exhibit distinct photochemical properties. A much better understood example of multiple LOV domain-containing BL photoreceptor is PHOT. It is known that the two LOV domains of PHOT divide into two clades in phylogenetic analysis (Crosson et al. 2003 and Fig. 2b), and they have different physiological roles in phototropism (Cho et al. 2007).

The distribution of helmchrome in swarmers of the Stramenopiles

Using anti-α-tubulin, anti-helmchrome antibodies and DAPI, we investigated the distribution of helmchrome in 40 swarmers from 37 species, of which 31 belongs to the

Stramenopiles. The presence or absence of helmchrome as well as other features including the presence/absence of the autofluorescence of PF, the PFB, the eyespot apparatus and the phototactic behavior are summarized in Table 1.

Brown algal swarmers released from species belonging to the Ectocarpales (plurispores of *E. siliculosus* and *Colpomenia bullosa*; zoospores of *Melanosiphon intestinalis*; male and female gametes of *S. lomentaria*, plurispores of *L. difformis*), the Cutleriales (female gametes of *Culteria cylindrica*), Desmarestiales (zoospores of *D. ligulata*), and the Ralfsiales (zoospores of *A. japonicas*) exhibit phototaxis, and all the swarmers have phototaxis-associated features such as the PF autofluorescence, the PFB structure, and the eyespot apparatus (Table 1). Helmchrome is localized along the entire length of the PF with a considerable accumulation at the PFB (Fig. 3a–e and Fig. S1a–c). This staining pattern of helmchrome resembles the distribution of the green autofluorescence that observed under blue violet light (Fig. 1 in Fu et al. 2014), indicating that helmchrome is the candidate of the autofluorescent substance.

Sperms of three species of Fucales show different results. Helmchrome was detected in *Fucus distichus* but not in *S. babingtonii* and *S. confusum* (Fig. 3f, g and Fig. S1d). This is consistent with that sperm of *F. distichus* possesses PFB structure and eyespot apparatus and show phototaxis (Müller et al. 1987), while in the latter two species, helmchrome could not be observed and the PF indeed have no autofluorescence and lack the PFB structure nor the eyespot apparatus (Table 1). The phototactic behavior of *S. confusum* and *S. babingtonii* sperms were not well determined due to their hardly releasing from the receptacles. Unlike swarmers of Ectocarpales and Cutleriales, the PF of *F. distichus* sperm is longer than the AF. In addition to the morphological differences, the staining pattern of the anti-helmchrome antibody appeared much more compact in the PF of Ectocarpales and Cutleriales swarmers, while a slightly punctate staining pattern was observed in the PF of *F. distichus* (Fig. 3f). This agrees with the fact that the autofluorescence of *F. distichus* PF is much weaker and will be easily bleached by BV irradiance while the PFs of Ectocarpales and Cutleriales swarmers can retain their green autofluorescence for a longer time.

C. filum is a filamentous brown alga belonging to the Laminariales. Although the staining on the cell body might be unspecific, the AF remained unstained indicating that helmchrome was stained in the PF and concentrated at the PFB (Fig. S1e). Zoospores of *C. filum* show phototaxis and possess phototactic structures, the PFB, and the eyespot apparatus (Table 1). However, swarmers of six other Laminariales species (zoospores of *S. japonica*, *S. angustata*, *U. pinnatifida*, *A. crassifolia* and *A. clathratum*, and sperm of *S. japonica*) do not show phototaxis. Helmchrome could not be detected in the PF of these swarmers (Fig. 3h, i and Fig. S1f–i), and the PF autofluorescence, the PFB structure, and the eyespot apparatus are absent (Table 1).

In addition to the brown algal species, swarmers of four other classes of the Stramenopiles were examined. Helmchrome was detected in zoospores of *S. ischiensis* (Fig. 3j), which is the only species of the Schizocladiophyceae class that shows a close phylogenetic relationship to the Phaeophyceae (Kawai et al. 2003). The staining pattern also resembles that of the brown algal swarmers. In the four unicellular Chrysophyceae species, the anti-helmchrome antibody labeled the PF of *O. danica* and *D. sertularia*, but not those of *D. sociale* and *S. petersenii* (Fig. 3k, l and Fig. S1j–k). The PFB structures were discernable in the PF of *O. danica* and *D. sertularia* under immunofluorescence microscopy, but the labeling with the anti-helmchrome antibody was weak. Coleman (1988) reported that the distribution of green autofluorescence in the PF of Chrysophyceae cells varied depending on the species that green autofluorescence was observed throughout the PF, restricted to the PFB structure or absent. This variation in autofluorescence distribution may explain why we could not detect helmchrome in *D. sociale* although it has similar phototactic features to *D. sertularia*. In contrast, *S. petersenii* lacks the eyespot apparatus and the autofluorescence of shorter flagellum; thus, the missing of these features agrees with the lack of helmchrome.

O. majus is a unicellular species of Xanthophyceae that releases flagellated reproductive zoospores (Pecora and Rhodes 1973; Lokhorst and Star 2003). Helmchrome can be detected in the PF with a strong fluorescence in the basal part (Fig. 3m). The staining pattern also resembles that of phototactic brown algal swarmers. Since zoospores of *O. majus* settled down onto the substratum immediately after release, we failed to determine its phototactic response; neither a previous literature describing phototactic behavior of this species is available.

The Labyrinthulomycetes are another subgroup of the Stramenopiles that produce biflagellate zoospores. Amon and French (2004) reported that zoospores of a thraustochytrid species, *Ulkenia* sp., showed positive phototaxis to blue light. We examined swarmers of five thraustochytrid species including *Ulkenia* sp. in this study (Table 1). Helmchrome was not detected in any of the swarmers of these species (Fig. 3n, o and Fig. S1l–o) nor was autofluorescence of the PF observed under BV irradiance. Amon and Perkins (1968) also reported that the eyespot apparatus and the PFB structure are not present in the Labyrinthulomycetes cells. Given the structural differences, it is possible that the photoreceptive process of the Labyrinthulomycetes swarmers might be different from that of PFB eyespot-bearing cells in the Stramenopiles.

Table 1 Distribution of helmchrome in various algal species

Group	Species	Type of swarmers	Helmchrome	Autofluorescence of PF	Paraflagellar body	Eyespot	Phototaxis	References*
Phaeophyceae	*Ectocarpus siliculosus*	Plurispore	+	+	+	+	+	Müller et al. (1987), Kawai (1988)
Phaeophyceae	*Colpomenia bullosa*	Plurispore	+	+	+	+	+	
Phaeophyceae	*Analipus japonicus*	Zoospore	+	+	+	+	+	Kawai (1988)
Phaeophyceae	*Scytosiphon lomentaria*	Male and female gametes	+	+	+	+	+	Kawai (1988)
Phaeophyceae	*Melanosiphon intestinalis*	Zoospore	+	+	+	+	+	Kawai (1988)
Phaeophyceae	*Leathesia difformis*	Plurispore	+	+	+	+	+	Kawai (1988)
Phaeophyceae	*Culteria cylindrica*	Female gamete	+	+	+	+	+	Kawai (1988)
Phaeophyceae	*Desmarestia ligulata*	Zoospore	+	+	+	+	+	Kawai (1988)
Phaeophyceae	*Desmarestia ligulata*	Sperm	−	−	−	−	−	
Phaeophyceae	*Fucus distichus*	Sperm	+	+	+	+	+	Müller et al. (1987), Kawai (1988)
Phaeophyceae	*Silvetia babingtonii*	Sperm	−	−	−	−	ND	
Phaeophyceae	*Sargassum confusum*	Sperm	−	−	−	−	ND	
Phaeophyceae	*Chorda filum*	Zoospore	+	+	+	+	+	Müller et al. (1987), Kawai (1988)
Phaeophyceae	*Saccharina angustata*	Zoospore	−	−	−	−	−	Kawai (1988)
Phaeophyceae	*Saccharia japonica*	Zoospore	−	−	−	−	−	Kawai (1988)
Phaeophyceae	*Saccharia japonica*	Sperm	−	−	−	−	−	Kawai (1988)
Phaeophyceae	*Agarum clathratum*	Zoospore	−	−	−	−	−	
Phaeophyceae	*Alaria crassifolia*	Zoospore	−	−	−	−	−	Kawai (1988)
Phaeophyceae	*Undaria pinnatifida*	Zoospore	−	−	−	−	−	Kawai (1988)
Schizocladiophyceae	*Schizocladia ischiensis*	Zoospore	+	+	+	+	+	Kawai (1988)
Chrysophyceae	*Ochromonas danica*		+	+	+	+	+	Kawai and Inouye (1989)
Chrysophyceae	*Dinobryon sertularia*		+	+	+	+	+	Müller et al. (1987)
Chrysophyceae	*Dinobryon sociale*		−	−	+	+	+	Müller et al. (1987)
Chrysophyceae	*Synura petersenii*		−	−	+	−	ND	Kawai and Inouye (1989)
Xanthophyceae	*Ophiocytium maius* zoospore		+	+	+	+	ND	Kawai and Inouye (1989), Lokhorst and Star (2003)
Labyrinthulomycetes	*Thraustochytrid-like* 12B		−	−	−	−	−	Perveen et al. (2006)
Labyrinthulomycetes	*Sicyoidochytrium* sp		−	−	−	−	−	
Labyrinthulomycetes	*Ulkenia* sp.		−	−	−	−	+	Amon and French (2004)
Labyrinthulomycetes	*Ulkenia amoeboidea*		−	−	−	−	+	Amon and French (2004)
Labyrinthulomycetes	*Parietichytrium sarkarianum*		−	−	−	−	−	
Labyrinthulomycetes	*Parietichytrium* sp.		−	−	−	−	−	
Prymnesiophyceae	*Chrysochromulina hirta*		−	−	−	−	ND	Kawai and Inouye (1989)
Chromalveolata	*Symbiodinium* sp.		−	−	−	+	+	
Chromalveolata	*Symbiodinium* sp.		−	−	−	+	+	

Table 1 (continued)

Group	Species	Type of swarmers	Helmchrome	Autofluorescence of PF	Paraflagellar body	Eyespot	Phototaxis	References*
Chromalveolata	*Bysmatrum* sp.		–	–	–	–	–	
Chromalveolata	*Gymnodinium* sp.		–	–	–	–	ND	
Ulvophyceae	*Ulva pertusa*	Male and female gamete	–	–	–	+	+	
Ulvophyceae	*Bryopsis plumosa*	Female gamete	–	–	–	–	+	

"+" indicates presence; "–" indicates absence or none

*In addition to the present study, related references are listed

ND not determined

Helmchrome-detectable species also bear a PFB-eyespot complex

Helmchrome can only be detected in the stramenopile swarmers that possess a PFB-eyespot complex. In brown algal swarmers, it has been observed that the PFB fits exactly into a concaved depression of the chloroplast, where eyespot globules are aggregated (Müller et al. 1987; Kawai et al. 1990; Maier 1997a, b). Rapid freezing fixation and freeze substitution approaches revealed the fine structure of brown algal PFB composing of two distinct areas filled with electron-dense materials and crystalized materials within the compartment between flagellar membrane and the axoneme (Fig. 4a and Fu et al. 2013). Immuno-electron microscopy shows that helmchrome is localized at the area of crystalized materials that directly face the eyespot apparatus (Fu et al. 2014). A thin section cut along the axonemal axis of the PF shows that the entire PFB structure is enclosed by hexagon-shaped eyespot granules (Fig. 4b). Thereby, the staining pattern of anti-helmchrome antibody concentrating at the PFB structure (Fig. 4c) indicates a close association between helmchrome and the eyespot apparatus. The function of brown algal eyespots is thought to reflect and focus light onto the PFB during phototaxis (Kawai et al. 1990; Kreimer et al. 1991). This steric arrangement of helmchrome and the eyespot granules may allow the transmission of an amplified signal of light stimulus from the eyespot to the PFB, thus enhance light-sensing efficiency for phototaxis. The PFB-eyespot complex is conserved only in the phototactic swarmers, but not in the non-phototactic cells of the stramenopiles (Fig. 4d–g). In *Euglena*, a "two-instant" photoreceptive mechanism exists in the phototactic responses, which demonstrate the important roles of PFB and eyespot apparatus functioning as light sensing structure and shading device, respectively (Lebert 2001). Iseki et al. (2002) verified that the photoreceptor responsible for phototaxis of *E. gracilis* is localized at the PFB. Ultrastructural studies have shown that both the crystalized material in the PFB of *E. siliculosus* and the paracrystalline material in the paraflagellar rod of *E. gracilis* are forming structures arranged in highly order (Fu et al. 2013; Hyams 1982; Moestrup 1982). Given the reflecting function of brown algal eyespot apparatus and the structural similarity with that of *E. gracilis*, it is speculated that a two-instant mechanism may also exist in phototactic brown algal swarmers, which further suggest that helmchrome might be the photoreceptor responsible for phototaxis.

Non-stramenopile swarmers do not possess helmchrome

In the present study, helmchrome could not be detected in swarmers of non-stramenopiles including four dinoflagellates, two green algae, and one haptophyta alga (Fig. S1p–w). It has been reported that there is a wide variety of eyespot

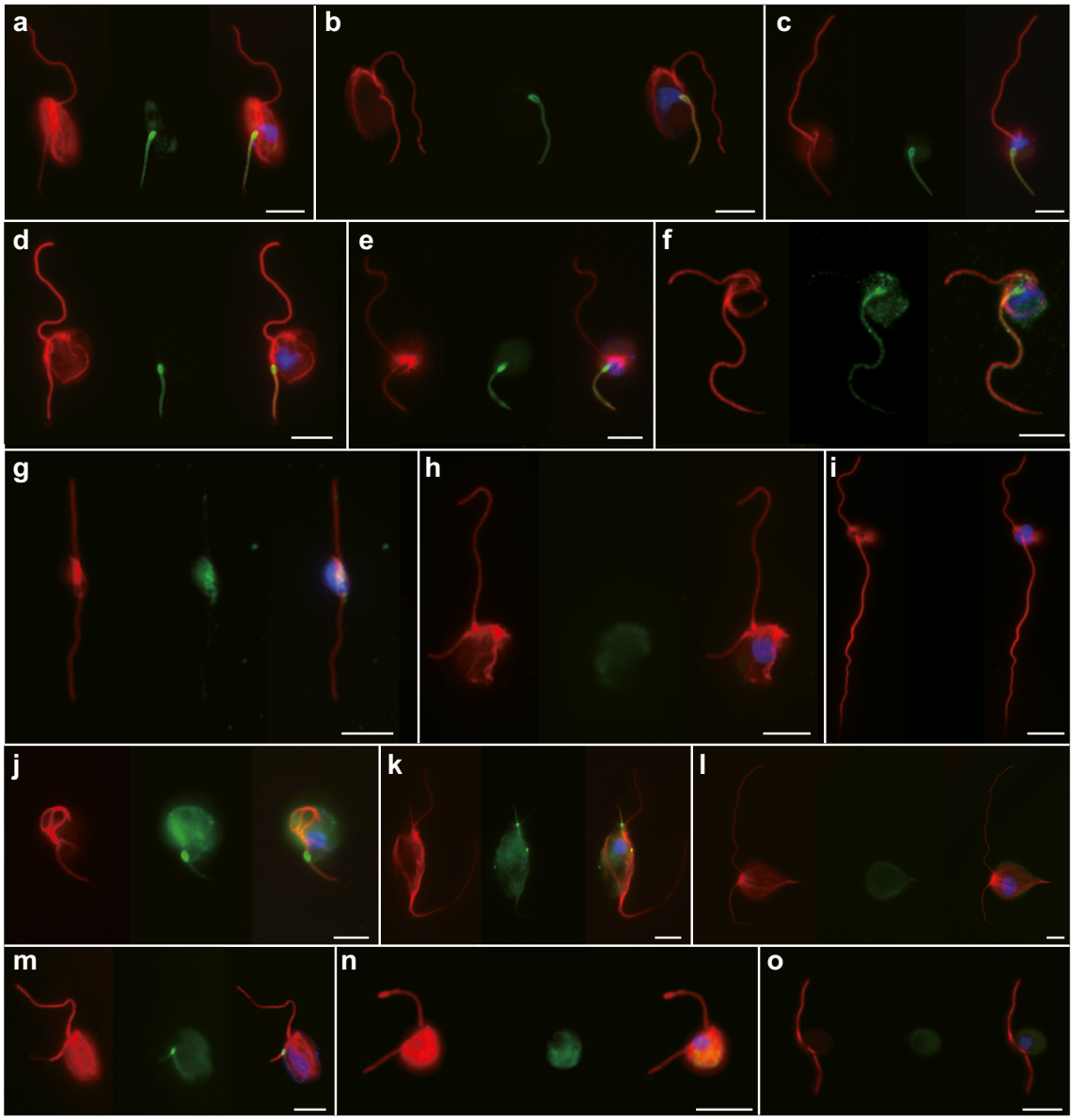

Fig. 3 Immunofluorescence microscopy images of 15 stramenopile swarmers including nine brown algal (**a**–**i**) and six non-brown algal (**j**–**o**) species. Images of α-tubulin (*red*), helmchrome (*green*) and merged with DAPI (*blue*) are shown for each sample. The cells are orientated with the AF extending upwards and the PF extending opposite. **a** Zoospore of *Ectocarpus siliculosus*. **b** Zoospores of *Melanosiphon intestinalis*. **c** Male gamete of *Scytosiphon lomentaria*. **d** Plurispores of *Leathesia difformis*. **e** Zoospore of *Analipus japonicas*. **f** Sperm of *Fucus distichus*. **g** Sperm of *Sargassum confusum*. **h** Zoospore of *Saccharina japonica*. **i** Sperm of *Saccharina japonica*. **j** Zoospore of *Schizocladia ischiensis*. **k** *Ochromonas danica*. **l** *Synura petersenii*. **m** Zoospore of *Ophiocytium majus*. **n** Zoospore of *Ulkenia* sp. **o** Zoospore of *Parietichytrium sarkarianum. Scale bars*, 5 μm

morphology and multiple light sensing systems existing in the dinoflagellate cells (Dodge 1984; Liu et al. 1990; Horiguchi et al. 1999). The two species examined in the present study, *Symbiodinium* sp. and *Symbiodinium* sp., possess eyespots and exhibit phototaxis; however, neither flagellar autofluorescence nor a PFB structure was observed. Kawai and Inouye (1989) reported that these features might be absent from the dinoflagellates. Although putative photoreceptor pigments responding to red light (Liu and Häder 1994) or a cryptochrome blue light receptor with a possible role in circadian control of the cell cycle (Brunelle et al. 2007) have been reported, no photoreceptor for phototaxis has been identified in dinoflagellates.

The male and female gametes of green algae *U. pertusa* and female gametes of *B. plumosa* show phototaxis and possess eyespot structures, but helmchrome, flagellar autofluorescence, and the PFB structure were not observed. The steric localization of the green algal eyespot (Fig. S2) is different from that of the phototactic brown algal swarmers. These different properties indicate that green algae and brown algae

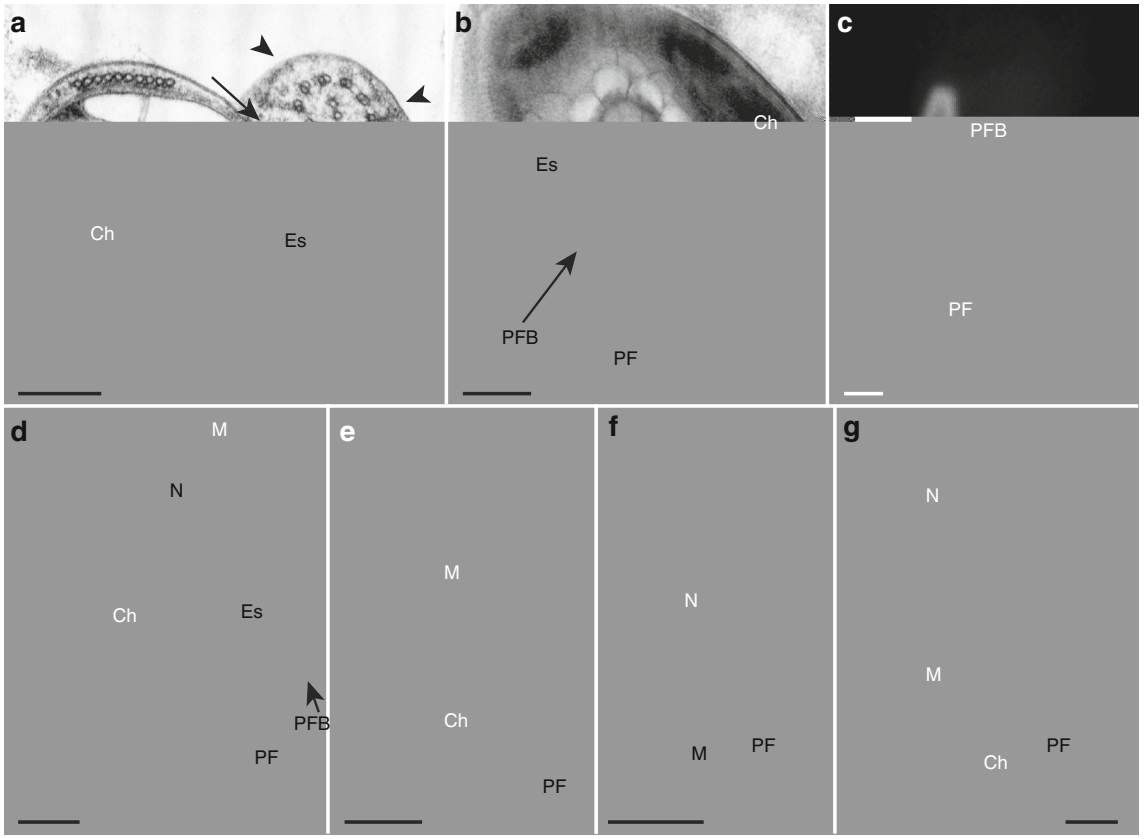

Fig. 4 Ultrastructure of the PFB-eyespot complex. **a** Cross-section view of the PFB-eyespot complex in a zoospore of *Colpomenia bullosa*. Crystalized material (*arrow*) and electron dense material (*arrowheads*) are indicated. **b** Longitudinal view of the PFB-eyespot complex in a male gamete of *Scytosiphon lomentaria*. Note that the PFB is enclosed by hexagon-shaped eyespot granules. **c** Immunofluorescence microscopy image of a male gamete of *Scytosiphon lomentaria* showing the distribution of helmchrome. **d**–**g** TEM images of four stramenopiles swarmers. The PFB-eyespot complex is conserved in zoospores of *Colpomenia bullosa* (**d**) but absent from zoospores of *Saccharina angustata* (**e**), *Thraustochytrid-like* 12B, and sperm of *Sargassum confusum* (**g**). *Ch* chloroplast, *Es* eyespot, *M* mitochondrion, *N* nucleus, *PF* posterior flagellum, *PFB* paraflagellar body. *Scale bars*, 200 nm in (**a**) and (**g**); 500 nm in (**b**) and (**d**–**f**); 1 μm in (**c**)

have evolved distinct phototactic systems to respond to light stimulation. In *Chlamydomonas*, the photoreceptor channelrhodopsins are light-gated ion channels localizing at eyespot apparatus, and they could trigger a cascade of transmembrane currents upon light stimulation, which eventually lead to a change in the flagellar beat pattern (Sineshchekov et al. 2002; Berthold et al. 2008). However, helmchrome of brown algae is localized within the compartment between axoneme and flagellar membrane, which could sense the light and subsequently transduce the signal to axonemal components without penetrating any membrane structure. The RGS domains of helmchrome also indicate that a signaling pathway involving G protein might exist in brown algae.

The phototactic behavior of the haptophyta alga *C. hirta* has not been previously investigated. In this study, the autofluorescence of flagella, this alga under BV irradiance was not observed. However, Kawai and Inouye (1989) reported the presence of autofluorescence in the proximal part of one flagella in this species. This disagreement is likely due to different cell growth conditions.

Helmchrome and aureochrome in the stramenopile group

Both helmchrome and aureochrome are proteins identified in the stramenopile organisms; however, they may play different roles in the spectrum of physiological activities. Although the present study demonstrated a wide distribution of helmchrome in various stramenopile species, the orthologous of helmchrome was not found by a reciprocal BLAST search in the available genome database of the Stramenopiles, which include pelagophytic alga *Aureococcus anophagefferens*, centric diatom *Thalassiosira pseudonana*, pennate diatom *Phaeodactylum tricornutum*, and a non-photosynthetic oomycete *Phytophthora infestans* (Fu et al. 2014). However, AUREO-like sequences were found in the genome database of these organisms except *P. infestans* (Ishikawa et al. 2009). Experimental data have proved that AUREO is involved in BL-induced branching of the Xanthophycean algae *Vaucheria frigida* (Takahashi et al. 2007) and light-dependent cell cycle onset of the diatom *Phaeodactylum* (Huysman et al. 2013). Because helmchrome is restricted to the PF or shorter flagellum of the phototactic swarmers that also bear the PFB-

eyespot complex, the absence of helmchrome sequence in the available stramenopiles genome can be explained by the fact that none of these organisms have flagella nor PFB-eyespot complex in their flagellated cells. For example, neither *V. frigida* nor *P. tricornutum* has flagellated cell stage; the sperm of centric diatom *T. pseudonana* has a reduced 9+0 flagellum lacking central pair microtubules (Idei et al. 2013); zoospore of oomycete *P. infestans* has two laterally inserted 9+2 flagella but no PFB-complex structure (Walker and van West 2007). It is speculated that aureochrome and helmchrome might play different physiological roles in the organisms of the Stramenopiles, that aureochrome functions in BL-induced morphogenesis and cell cycle, while helmchrome is possibly involved in phototaxis of the flagellated swarmers due to its exclusive distribution among the swarmers showing phototaxis.

Conclusion

Our data reveal that helmchrome is widely conserved in phototactic swarmers of the Stramenopiles and is specifically localized at the PF with dense distribution at the PFB structure. Swarmers possessing helmchrome exhibit similar physiological and structural features such as green autofluorescence of the PF and presence of the PFB-eyespot complex. This suggests that phototactic swarmers of the Stramenopiles might share a common photoreception system. We propose that helmchrome is the photoreceptor involved in phototaxis of these swarmers. Further investigations on the function of helmchrome using techniques like RNAi will be necessary for a better understanding of this protein.

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Conflict of interest The authors declare that they have no conflict of interest.

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