UV-green dichromacy in the basal hymenopteran *Sirex noctilio* (Hymenoptera: Siricidae).

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Abstract

A precondition for colour vision is the presence of at least two spectral types of photoreceptors in the eye. The order Hymenoptera is traditionally divided into the Apocrita (ants, bees, wasps) and the Symphyta (sawflies, woodwasps, horntails). Most apocritan species possess three different photoreceptor types. In contrast, physiological studies in the Symphyta have reported one to four photoreceptor types. To better understand the evolution of photoreceptor diversity in the Hymenoptera, we studied *Sirex noctilio*, which belongs to the superfamily Siricoidea, a sister group of the Apocrita. Our aim was to i) identify the photoreceptor types of the compound eye by electroretinography (ERG), ii) characterise the visual opsins genes of *S. noctilio* by genomic comparisons and phylogenetic analyses and iii) analyse opsin mRNA expression. ERG measurements revealed two photoreceptor types in the compound eye, maximally sensitive to 527 and 364 nm. In addition, we identified three opsins in the genome, homologous to the hymenopteran LW1, LW2 and UV opsin genes. The *LW1* and *UV* opsins were found to be expressed in the compound eyes, and *LW2* and *UV* opsins in the ocelli. The lack of a SW-homologous opsin gene and a corresponding receptor suggests that *S. noctilio* is a UV-green dichromate.

Introduction

The ability to see colours, i.e. to discriminate between different wavelengths of light independent of intensity, provides insects with valuable information about their environment. Visual information, including shape, movement and colour of visual stimuli can induce adaptive behavioural responses. Dragonflies possess reduced antenna and auditory organs but well developed and enlarged compound eyes to visually locate their prey and mate[1]. Visual stimuli can also be important for mate recognition[2], mate choice[3,4], location of food[5,6] or avoidance behaviour[3]. Insect colour vision may also play a role in more complex tasks such as counting[7] and social learning[8]. Colour vision requires the presence of at least two different photoreceptor types tuned to different wavelengths of the light spectrum[9,10]. Sensitivity to different wavelengths can be under selective pressure and consequently varies among insects with different visual ecology[11–13].

The sensitivity of each photoreceptor is determined by its light-sensitive visual pigment. The pigment consists of the opsin protein and a vitamin A-based chromophore[14]. The sensitivity of the chromophore to different wavelengths can be tuned by the opsin protein, which is called spectral tuning. There are three major groups of visual opsin genes in insects, named for the region of the spectrum they absorb; the ultraviolet (UV), blue or short wavelength (SW) and green or long wavelength (LW) sensitive opsins[9]. The LW group in Hymenoptera can be subdivided into the LW1 and the basal LW2 opsins, expressed in the compound eyes and the ocelli, respectively[15].

Colour vision appears to differ in the Apocrita and Symphyta. All Apocrita investigated to date, except for the Ichneumonidae, possess one copy of each visual opsin gene[16–18]. In contrast, between one and four photoreceptor types have been reported in the Symphyta[16]. For example, evidence for an additional

(fourth) red photoreceptor was found in three species but not in others. On the other hand, the SW seems to be missing in all but one species. More recent studies assumed that the SW photoreceptor was present but not found experimentally[9]. To date, there is no clear evidence for the presence or absence of SW opsins in the Symphyta. It is also not clear what mechanism could have driven opsin gene loss and gain in the basal Hymenoptera.

The biology of the diurnal woodwasp *S. noctilio* (Symphyta) suggests an important role of vision. There is a strong sexual dimorphism, where males are black with an orange abdomen and females are completely black with steel blue iridescence (from a human perspective). Field studies have reported that males aggregate at the top of pine trees to form leks and females are attracted to these for mating[19–21]. A male specific putative pheromone released from the sexually dimorphic hind legs has been described[22]. We refer to this as a putative pheromone because although attraction was observed in the lab[23], it was inactive in field trials[24]. In addition, a study of flight distribution showed that females exhibit a higher flying altitude when males are present compared to when males are absent[25]. A field trapping study observed that simulated leks, where traps were baited with dead males, increased the number of females captured in traps, potentially due to visual signals[26]. Finally, the addition of UV light greatly increased the number of *S. noctilio* captured in different trap types[27]. Despite these indications of the importance of visual stimuli on the behaviour of the woodwasp, the visual ecology of *S. noctilio* is poorly understood.

The evidence suggesting an important role for (colour) vision, the potential lack of a SW opsin in the Siricidae and the phylogenetic position of *S. noctilio* between the tetrachromatic *Tenthredo campestris* and the trichromatic Apocrita[9,16], makes it a promising model to understand the evolution of colour vision in basal Hymenoptera. In this study, we i) determined the spectral sensitivity of the compound eyes of *S. noctilio* by means of electroretinography, ii) characterised the diversity of visual opsin genes using genomic tools and iii) analysed opsin expression in the compound eyes and ocelli of *S. noctilio*.

Results

ERG

Ten females and ten males were measured under dark adaptation (Fig. 1a). The λ_{max} of the LW receptor was determined to be λ_{max} = 527 ± 2 nm (residual standard error = 0.04299, Student t-test p-value = 6.18×10^{-10}). Eight females and seven males were measured under dim green light adaptation (Fig. 1b). No evidence of a potential SW (blue) receptor was visible when both the UV- and LW-light adapted models were fitted to the responses of the dark-adapted eye. Electroretinogram recordings were made from the central part of the eyes of seven female and seven male *S. noctilio* under strong green light adaptation (Fig. 1c). These recordings were used to determine the λ_{max} of the UV photoreceptors with minimal contribution of the LW receptors. The λ_{max} of the UV receptor under strong green light adaptation was found to be λ_{max} = 364 ± 3 nm (residual standard error = 0.121, Student t-test p-value < 2×10^{-16}).

Genetic analyses

Three putative visual opsin genes were found in the genome of *S. noctilio*. The BLAST analyses (Table 3, Supplementary material) revealed that genes on the scaffolds 6, 7 and 692 of the *S. noctilio* genome assembly were the best matches for the UV, LW1 and LW2 opsin genes from both *A. mellifera* and *O. abietinus*. After curation, the genes in the genomic sequences consisted of 1658, 2549 and 2381 nucleotides, respectively, for the putative UV, LW1 and LW2 opsins. The peptide sequences were made of 371 amino acids including five exons for the putative UV opsin, 383 amino acids including five exons for the putative LW1 opsin and 400 amino acids including eight exons for the putative LW2 opsin. Visual opsin genes identified in this study were deposited in the NCBI library under the accession numbers MW340972 - MW340974.

A total of 209 visual opsin sequences were found in 78 hymenopteran species including 77 LW1, 43 LW2, 43 SW, and 46 UV opsins (Table 4, Supplementary material). In 42 species, the complete pool of visual opsin were located because the genome was available or the opsins were extracted. These 42 species show few variations within opsin groups, where 41 LW1, 40 LW2, 41 SW and 42 UV were found. All possess a copy of the UV opsin. The European woodwasp, *S. noctilio*, is the only species lacking the SW opsin. Two species of ants, *Cardiocondyla obscurior* and *Cerapachys biroi*, are the only two species lacking the LW2 opsin. One species of ant, *Camponotus rufipes*, did not have a copy of the LW1 opsin. The remaining 36 species thought to have an incomplete pool of opsins includes 28 Myrmecia species, *Nothoyrmecia macrops, Osmia rufa*, *Diadasia afflicta*, *Diadasia rinconis*, *Camponotus atriceps* and *Cataglyphis bombycinus*, from which only the LW opsins were extracted, and *Tenthredo koehleri* and *Chrysis viridula*, which lack the LW2 opsin.

The phylogenetic analysis (Fig. 2) of the visual opsin peptide sequences in Hymenoptera resolved four clearly separated clades representing the different opsin groups (SH-aLRT ≥ 80 % and UFboot ≥ 95 %). The UV opsins grouped sister to the SW opsins group. The two clades for the two LW opsins group together. The first LW opsin clade contains the LW2 opsins, the second clade contains the LW1 opsins.

The translated sequences of the three putative visual opsin genes in *S. noctilio* fall within three different groups. The putative visual opsin sequences found on scaffold 6, 7 and 692, respectively, fall within the UV, LW1 and LW2 opsin groups. The UV opsin sequence of *S. noctilio* groups with *O. abietinus* (Symphyta), and is closely related to the *Cephus cinctus* (Symphyta) UV opsin sequence. The LW1 opsin sequence in *S. noctilio* groups most closely with the LW1 opsin sequence of the Vespoidea (Apocrita) and Chalcidoidea (Apocrita) species. The LW2 opsin sequence in *S. noctilio* groups most closely with the LW2 opsin sequence of the Tenthredinoidea (Symphyta) species.

No SW opsin gene was found in the genome of *S. noctilio* (Fig. 3). No high-scoring matches were obtained from the BLAST search analyses of the SW opsin genes from other Hymenoptera in the genome of *S. noctilio*. Local BLAST searches using the genes flanking the SW opsin gene in other Hymenoptera (including *A. mellifera*, *O. abietinus* and *C. floridanus*) as queries against the *S. noctilio* genome indicated that the *S. noctilio* orthologues of these flanking genes are located on scaffold 35 of the genome

assembly. Gene regions coding for Lar-Tyr phosphatase genes were found to be present downstream of the SW opsin gene in *A. mellifera* and *C. floridanus*. No Lar-Tyr phosphatase genes were found in *O. abietinus* likely because the available assembled scaffold ended after the SW opsin gene. Two genes were found upstream of the SW opsin gene in *A. mellifera*, *O. abietinus* and *C. floridanus*. These two genes code for a cell adhesion molecule and a Ser-Tyr kinase SBK1 gene, and both could be identified in *S. noctilio.* In addition, a gene coding for an unknown protein located between the cell adhesion molecule and the Ser-Tyr kinase SBK1 genes was observed in *O. abietinus*, *C. floridanum* and *S. noctilio*. The SW opsin gene in *S. noctilio* was not found between the Lar-Tyr phosphatase genes on one side and the cell adhesion molecule on the other side, as in other hymenopterans. No sequence similarities to other hymenopteran species could be identified when performing BLAST analyses of the genomic section where the SW opsin gene was expected to be found in *S. noctilio*. Assessment of genome sequence reads mapped back to the scaffold where the SW opsin was expected to be found indicated that the absence of this gene cannot be explained by incorrect assembly of the *S. noctilio* genome.

Transcriptome analyses

Trancriptome analyses show that the LW1, LW2 and UV genes are differentially expressed in the compound eyes and ocelli (Fig. 1, Supplementary material). The *LW1* gene was expressed in the compound eyes of both male and female woodwasps. In contrast, the *LW2* gene was expressed only in the ocelli of both sexes. The UV gene was the only opsin expressed in both the compound eye and the ocelli. Sequencing analyses of the expressed PCR product confirmed that the expected transcripts were amplified (Fig. 2, Supplementary material).

Discussion

Our ERG bioassays and transcriptomic analyses showed UV-green dichromacy in *S. noctilio*. Two photoreceptors were found in the compound eyes with peak maxima at λ_{max} = 527 ± 2 nm and 364 ± 3 nm, respectively. Consistent with previous studies, *LW1* was found to be expressed in the compound eyes, *LW2* was found to be ocelli specific and the *UV* opsin was found to be expressed in both compound eyes and ocelli as has been shown in bees[28]. The peak maxima in *S. noctilio* are similar to previous ERG studies on Symphyta (Siricoidea, Xiphidrioidea and Tenhredinoidea) where the SW opsin was also not found[16]. Later studies assumed that the SW photoreceptor was present but missed, or that the expression levels were much lower than for LW photoreceptors and therefore not detected[9].

We could not find any evidence of a SW opsin pseudo-gene in the genome of *S. noctilio*, suggesting that the complete loss of the SW opsin likely did not occur recently in *S. noctilio*, but at a higher phylogenetic level. We suggest that earlier studies may have failed to detect a SW photoreceptor not because of low expression levels, but rather because it has been lost by a common ancestor. The latest phylogeny for the Hymenoptera suggests that Siricoidea and Xiphidrioidea form a monophyletic group[29]. It is likely that the SW opsin has been lost in the common ancestor of both families. Physiological studies on two species of Tenthredinoidea and one species of Xiphidrioidea suggested that these families possess a red-sensitive

photoreceptor[16]. In the same study, a red photoreceptor was not found in the one species of Siricoidea tested (*Urocerus gigas*). The red photoreceptor was likely lost in the Siricoidea and later families, but might still be present in earlier diverged families such as Xiphidrioidea and Tenhredinoidea. Future studies need to investigate the loss and gain of photoreceptors and underlying opsins in the Symphyta to fully understand opsin evolution in these basal groups.

A UV-green dichromatic visual system might be advantageous during mate searching. Insects such as bees and dragonflies sometimes also show functional dichromacy in particular parts of their compounds eyes[30]. For example, the dorsal area of the honeybee compound eye in drones only expresses UV and blue-sensitive photoreceptors, most likely to facilitate efficient detection of queens against the bright sky. In contrast, honeybee workers possess three receptors in the entire eye (except for the dorsal rim area) to facilitate flower recognition[28,31]. In dragonflies, both sexes possess a region of UV-blue dichromacy in the dorsal part of the eye to increase their ability to detect small prey items against the blue sky when hunting[32,33]. It is not clear if opsin expression differs between the dorsal and the ventral region of the compound eye of *S. noctilio*. However, adult *S. noctilio* do not feed, and thus have no need for flower recognition or detection of other food sources[34]. UV-green dichromatism has been shown to be more reliable than blue-green dichromatism for distinguishing foreground objects against the sky[35]. In *S. noctilio*, adults mate in the canopy of trees and thus it is likely that in *S. noctilio* dichromacy is superior to trichromacy for mate detection.

A dichromatic visual system might also be beneficial to avoid predators. Vertebrate dichromatic species can outcompete trichromatic species at foraging in shady environments, likely because they are more alert to achromatic cues[36–39]. Adult female *S. noctilio* spend a substantial amount of time under the canopy searching for suitable oviposition sites, where light can be limited. Dichromacy in this environment could increase the ability of females to avoid predation and locate appropriate oviposition sites.

The ocellar dichromacy observed in *S. noctilio* is well conserved in flying insects (e.g. Orthoptera[40], Odonata[1], Mantodea[41], Lepidoptera[42] and Hymenoptera (bees)[43]). In Hymenoptera, the presence of a LW ocelli specific opsin has been confirmed in bees[15,28], fig wasps[18], and in the Siricidae (this study). In contrast, ants only possess a UV sensitive opsin in their ocelli[44]. In some ants the ocelli have been linked to celestial compass orientation[36], where only a UV opsin may be enough for this purpose. For most flying hymenopterans (and possibly flying insects in general), dichromatic ocelli seem to provide an advantage for flight stabilisation. The UV-green contrast together with the triangular arrangement of the ocelli provides a solid skyline delimitation[45]. On flight mills *S. noctilio* has been observed to fly as much as 19–49 km in a 24 hr period[46,47]. A dichromatic ocellar system that enhances flight stabilisation is consistent with this high dispersal capacity.

This study characterized the visual system of S. noctilio at the genetic and physiological level and lays the foundation for further work on the visual systems of basal Hymenoptera. To our knowledge, *S.*

noctilio is the first confirmed case of true dichromacy (i.e., genetic and physiological evidence) in the Hymenoptera where the SW opsin gene has been lost. Tetra-, tri- and dichromacy has now been reported in the Symphyta. Our results provide support for the hypothesis that trichromacy is advantageous for processing visual inputs such as flowers and prey at ground level, whereas dicromacy is superior for detecting objects against the bright sky and for breaking camouflage in a low light environment. Studying opsin evolution in Symphyta, and particularly the loss and gain of SW and red photoreceptors, offers exciting opportunities to understand the ecological relevance of di-, tri- and tetra-chromacy in closely related families of insects.

Materials And Methods

Insects

Pine logs infested with *S. noctilio* were collected from Knysna (South Africa) (n = 133). Trees with characteristic symptoms of infestation, such as brown needles and fresh resin droplets oozing from the bark from oviposition sites were selected and cut into logs. Logs were stored in an insectarium at 20°C with ambient humidity and a photoperiod of 12/12 hr L/D. After emergence, insects were collected and stored in a fridge at 12°C for later use. Before any analyses were performed, insects were left under artificial light for 30 minutes at room temperature. The use of plants in this study complies with international, national and/or institutional guidelines.

Electroretinographical recordings (ERG)

To reduce external noise, recordings were conducted in a grounded Faraday cage painted completely black. Live, intact animals were fixed in a custom-made Plexiglas holder using dental wax. Antennae were carefully fixed to ensure they did not obstruct the measured compound eye. The reference electrode (25 µm silver wire) was inserted into the head capsule behind the compound eye. Before inserting the recording electrode, a little hole was drilled into the cuticle of the compound eye using a minute pin. Recording electrodes were glass capillaries pulled with a DMZ-Universal Puller (DMZ-Universal Puller, Zeitz-Instruments, Germany) filled with 1 molar Potassium-Chloride solution. To be consistent among insects, recording electrodes were always placed carefully at the centre of the compound eye just under the cuticle surface. The signal was 10x amplified (Neuroprobe Amplifier Model 1600, A-m System Inc. Sequim, USA) and Bandpass filtered 0.1–150 Hz using a dual variable filter (VBF 8, Kemo Inc., Greenville, USA). The filtered analogue signal was digitized using an analogue-digital receiver (Lap-Trax 4/16, World Precision Instruments, USA) to be visualized and recorded with the computer software LabScribe (LabScribe Version 3.010800, iWorx Systems Inc., USA).

ERG: Light stimulation

A xenon arc lamp (Abet Technologies Inc., Model LS-150-Xe SN 127, Milford, USA) was used to produce a full daylight spectrum with a spectral range from 320–820 nm. Optical band pass filters (Edmund Optics Inc., Barrington, USA) with a 20 nm full width-half maximum, produced the monochromatic light stimuli

tested. For each stimulus, light intensity was adjusted to $1.04 \pm 0.07 \times 10^{14}$ photons/cm^2 × sec using a motor driven neutral greyscale filter wheel (Nanotec-Munich, Model ST2818L1006B, Munich, Germany) controlled by an Arduino board. Two motor driven wheels (Lambda 10 – 2, Model LB10-2, Shutter Instrument, Novato, USA) equipped with the different band pass filters were positioned successively into the light beam. The first one included a shutter to produce light flashes of 100 ms. The Arduino, the shutter and the filter wheels could be controlled using a custom-made MatLab script (The MathWorks Inc., MatLab R2014a, Version 8.3.0.532). Having passed the computer controlled filter settings the light was guided through an optical quartz fibre placed 1 cm in front of the eye.

Before and after each series of spectral measurements, eight increasing intensities of white light were flashed for 100 ms on the insect eye. The range of white light intensity was generated by passing the light through a combination of eight positions of the neutral grey filter, which created intensities of white light that ranged from 5.97×10^{12} to 4.95×10^{16} photons/cm^2 × sec. After the last white light flash, insects were given 15 min to readapt to ambient light conditions before the first round of spectral measurements. For each series of spectral measurement, two rounds of 17 monochromatic lights (334, 352, 358, 382, 398, 418, 431, 449, 468, 501, 519, 529, 550, 569, 599, 620 and 649 nm) were flashed for 100 ms with 1 minute between each flash of monochromatic light. One minute after the first round ended, a second round of the same 17 monochromatic lights were flashed every minute in a different random order. This procedure was conducted for three light adaptations. Insects were first dark-adapted for 15 min prior to the experiment. When the last white flash from the first experiment was finished, insects were left for 15 min under a constant dim green light (551 nm at 7×10^{12} quanta/s/cm^2) and the procedure was repeated. The same procedure was used a third time under a constant strong green light (551 nm at 7×10^{15} quanta/s/cm^2). Insects were excluded from the dataset if no depolarisation was observed to the brightest white light.

ERG analyses

For each individual and under each light adaptation, a V-log(I) curve was computed. The depolarisation for each white light intensity flashed before and after the spectral measurements were averaged and a logarithmic model was used to create a V-log(I) curve. In order to start at log(0), the different intensities were log(I) transformed and the log(I) of the weakest value was subtracted from each white light intensity. The amplitude signal was transformed into the equivalent intensities log(I) for each monochromatic wavelength tested with the V-log(I) curve. The sensitivity (S) for each wavelength tested was computed using the method described in Telles *et al.* (2014)[48] with the following equation:

$$S(\lambda) = 10^{(\log(I) - \log(I_{max})}$$

where log(I) is the equivalent intensity for each response, and log(I_{max}) is the equivalent intensity of the highest response for each series. Sensitivity values were averaged for each sex under the same light adaptations. The difference in quantum catch for the receptor i ($\Delta(Qi)$) and a colour stimulus was calculated with the equation:

$$\Delta(Qi) = ki \times Ri(\lambda),$$

where $Ri(\lambda)$ is the sensitivity of the receptor i for the wavelength λ, and ki an arbitrary scaling factor for the photoreceptor i for each light adaptation [49]. The Ki for the UV peak under strong green light adaptation and for the LW1 photoreceptor under dark adaptation was defined as 1 since these adaptations are maximising the sensitivity of each photoreceptor. For each light adaptation, the sensitivity of the wavelength measured (Ri measured) at 364 nm for the UV and 527 nm for the LW1 was divided by the Ri measured under strong green light and dark adaptation, respectively.

Statistical analyses

The Stavenga 2 parameter opsin template[50] was used to determine the λ_{max} of each photoreceptor. The averaged sensitivity value for each receptor was fitted with the nlsLM function from the "minpack.lm" package in R (https://cran.r-project.org) into the different templates with the corresponding constant for each model[50]. Models were compared and the residual standard error and P-value were used as a measure to choose the best model fitting the data. For each light environment, no difference between male and female sensitivity was detected (Kruskall-Wallis test > 0.05 for both LW1 and UV). Therefore, data from both sexes were pooled and averaged for curve fitting.

Genetic analyses

Genomic coding sequences (CDS) of the visual opsin genes LW1, LW2, UV and SW of *Apis mellifera* (*Lop1*, *Lop2*, *Uvop* and *Blop*) and *Orussus abietinus* were used to perform a local BLASTp and BLASTn[51] search against the genome assembly and annotation of *S. noctilio* (Alisa Postma *et al.* unpublished data) using CLC Main Workbench V7.7.3 (www.clcbio.com). Sequences with an E-value <10^{-20} were retained for further analyses. The Apollo Genome Annotation and Curation tool[52] was used to curate the identified gene models.

The SW opsin gene was not identified using the above-mentioned BLAST searches; therefore, a targeted search for the SW opsin gene in *S. noctilio* was conducted. Local BLASTn, BLASTp and tBLASTn searches were performed using the SW opsin nucleotide and amino acid sequences from *A. mellifera*, *O. abietinus* and *Camponotus floridanus* species as queries against the genome assembly (nucleotide sequence) and the annotation (protein sequences) of *S. noctilio*. The flanking genes of the SW opsin gene from the genome of *A. mellifera*, *O. abietinus* and *C. floridanus* were identified and used to perform local BLAST searches against the *S. noctilio* genome. The corresponding flanking genes in *S. noctilio* were annotated. The section in scaffold 35 surrounded by the flanking genes in *S. noctilio* where the SW opsin

was found in other insects was used to perform local BLAST analyses on NCBI including all available hymenopteran genomes. In order to assess whether the absence of the SW opsin gene in the *S. noctilio* assembly could be ascribed to errors/misassembly of the genome sequence, the raw genomic sequencing reads used to generate the genome assembly of *S. noctilio* were mapped back to scaffold 35 from the *S. noctilio* genome using the Burrows-Wheeler Aligner (BWA)[53]. The resulting alignments were analysed and visualised using the Integrative Genomics Viewer (IGV)[54].

Hymenopteran visual opsin DNA, RNA and protein sequences were obtained from the literature and online resources including GenBank[55], OrthoDB[56] and i5K[57]. For these sequences, the gene predictor Augustus[58] was used to remove potential introns and to translate DNA sequences into amino acid sequences. RNA sequences were translated into amino acid sequences via MEGA7[59]. Amino acid sequences were aligned using MAFFT[60] under default parameters. Data were visually curated after alignment. Accession numbers of the hymenopteran sequences used for the phylogenetic reconstruction and aligned curated sequences are available (Table 1, Supplementary material).

Phylogenetic reconstruction was performed in IQTree v1.4.4[61]. The most likely amino acid substitution model was found to be the LG+F+I+G4. This model was used to build a Maximum Likelihood tree with 10,000 ultrafast bootstrap iterations[62] and 10,000 SH-like approximate likelihood ratio tests[63] were used to assess nodal support. The tree was rooted at the midpoint and visualised in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) .

Transcriptome analyses

RNA was extracted from both male and female *S. noctilio*. The compound eyes and the ocelli were surgically removed using a clean scalpel blade and stored individually in Eppendorf tubes at -80 °C. The compound eyes and ocelli of ten individuals of each sex were pooled, and a NucleoSpin RNA purification kit (Macherey-Nagel) was used to extract and purify the RNA. The quantity and quality of RNA was assessed by means of a nanodrop and a 2 % electrophorese gel, respectively. The RNA was then transformed into cDNA via a cDNA synthesis kit (SensiFAST).

Specific primers for *S. noctilio* were designed to amplify the LW1, LW2 and UV opsin genes in the Genscript primer design software (https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool) using default parameter values (Table 2, Supplementary material). Primers were designed with at least one intron present between the forward and reverse primer. Polymerase chain reactions (PCR) were performed for all samples and primers as follows: denaturation at 95 ºC for 5 min, followed by 40 cycles of 94 ºC for 30 s, 49 ºC for 30 s, 72 ºC for 1 min, then 7 min at 72 ºC. The PCR products were run on 2 % agarose gel at 110 V, 400 mA for 30 min. The PCR products were prepared for sequencing when the genes were thought to be expressed. A PCR clean-up was performed by adding 8 µL of Exo-SAP to the PCR product and put at 37 ºC for 15 min and 80 ºC for 15 min. A sequencing PCR was performed by adding 6.4 µL of dH20, 2.1 µL of sequencing buffer, 0.5 µL of BigDye, 1 µL of primer and 2 µL of cleaned PCR product. The PCR thermocycling profile used was 27 cycles of 96 ºC for 10 s, 55 ºC for 15 s, 60 ºC

for 4 min. Samples were subsequently washed and precipitated for sequencing. Samples were cleaned with 50 µL of 100 % EtOH, 2 µL of NaOAc and 8 µL of dH2O and centrifuged at 13400 rpm and 4 ºC for 30 min. The supernatant was removed and 150 µL of 70 % EtOH was added and the mixture centrifuged at 13400 rpm and 4 ºC for 10 min. This step was repeated a second time. The supernatant was removed and tubes were left open under the fume hood overnight to dry. Dried samples were sent for Sanger sequencing at FABI, University of Pretoria (South Africa). Sequencing results were manually curated in CLC Main workbench. Base calling conflicts were resolved by selecting the peak with the highest relative fluorescence units. Noise and contamination data under 4000 relative fluorescence units were eliminated. Sequences were aligned with the corresponding genomic sequence under default parameters.

Declarations

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Author contributions

QG carried out sequence alignments, transcriptomic, phylogenetic and electroretinogram analyses, statistical analyses, participated in the design of the study and wrote the manuscript; JS carried out the electroretinogram analyses, participated in the design of the study, participated in the genetic comparison and critically revised the manuscript; BS coordinated the study, participated in the design of the study and critically revised the manuscript; MS participated in the electroretinogram analyses and reviewed the manuscript; and JA coordinated the study, participated in the design of the study and critically revised the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

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Competing of interests

The author(s) declare no competing interests.

Data availability

https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/HCNMW4

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Figures

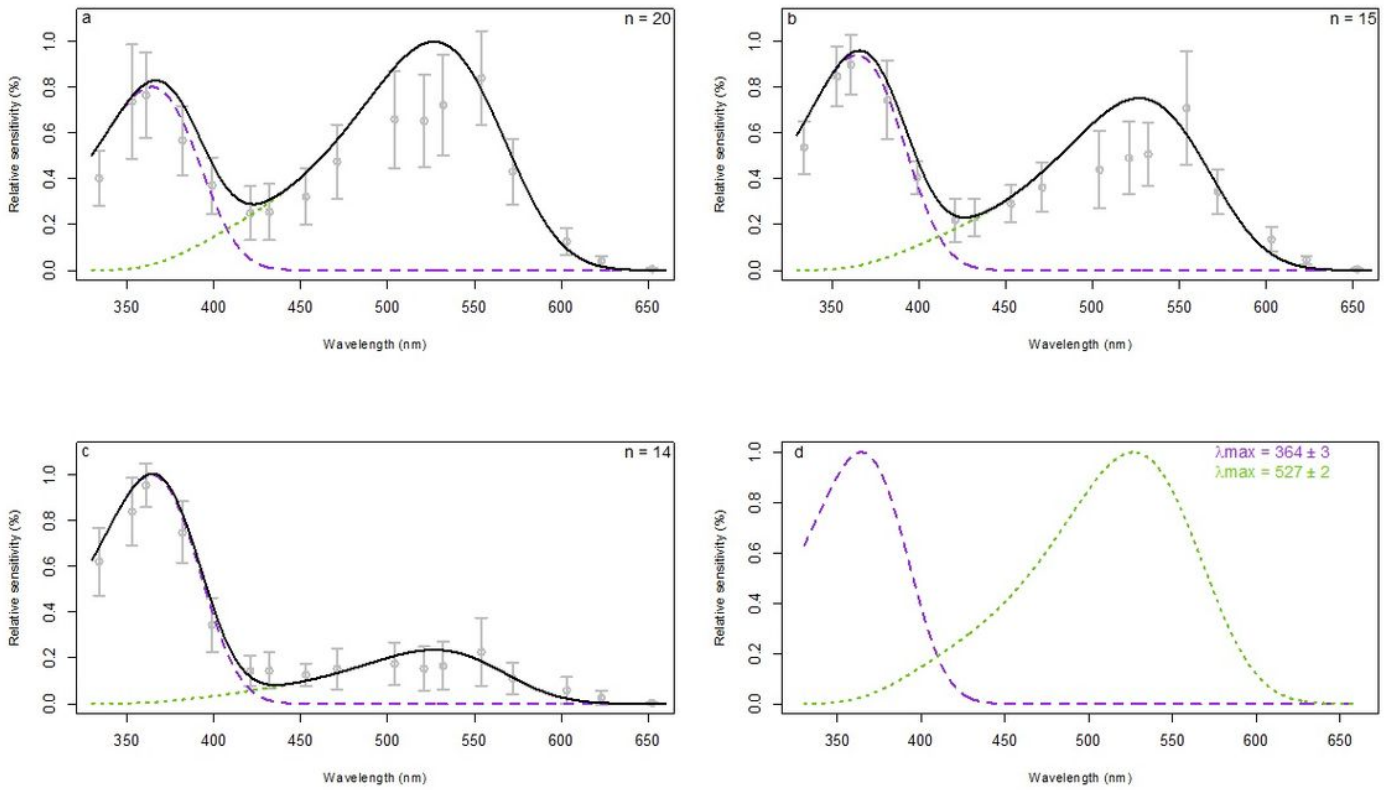

Figure 1

Spectral sensitivity of the compound eyes of S. noctilio determined by ERG after (a) dark adaptation (n = 10 males and 10 females), (b) dim green light adaptation (n = 7 males and 8 females), and (c) green light adaption (n = 7 males and 7 females). Normalized spectral sensitivity of the two types of receptors (d). Grey circles represent the relative sensitivity (%) measured from ERGs (mean ± standard deviation). Dashed (purple) and dotted (green) lines indicate the Δ(Qi) of the UV and LW receptors, respectively, under each adaptation. The black line shows the sum of the relative contribution of the UV and LW receptor model multiplied by the respective scaling factors for each adaptations.

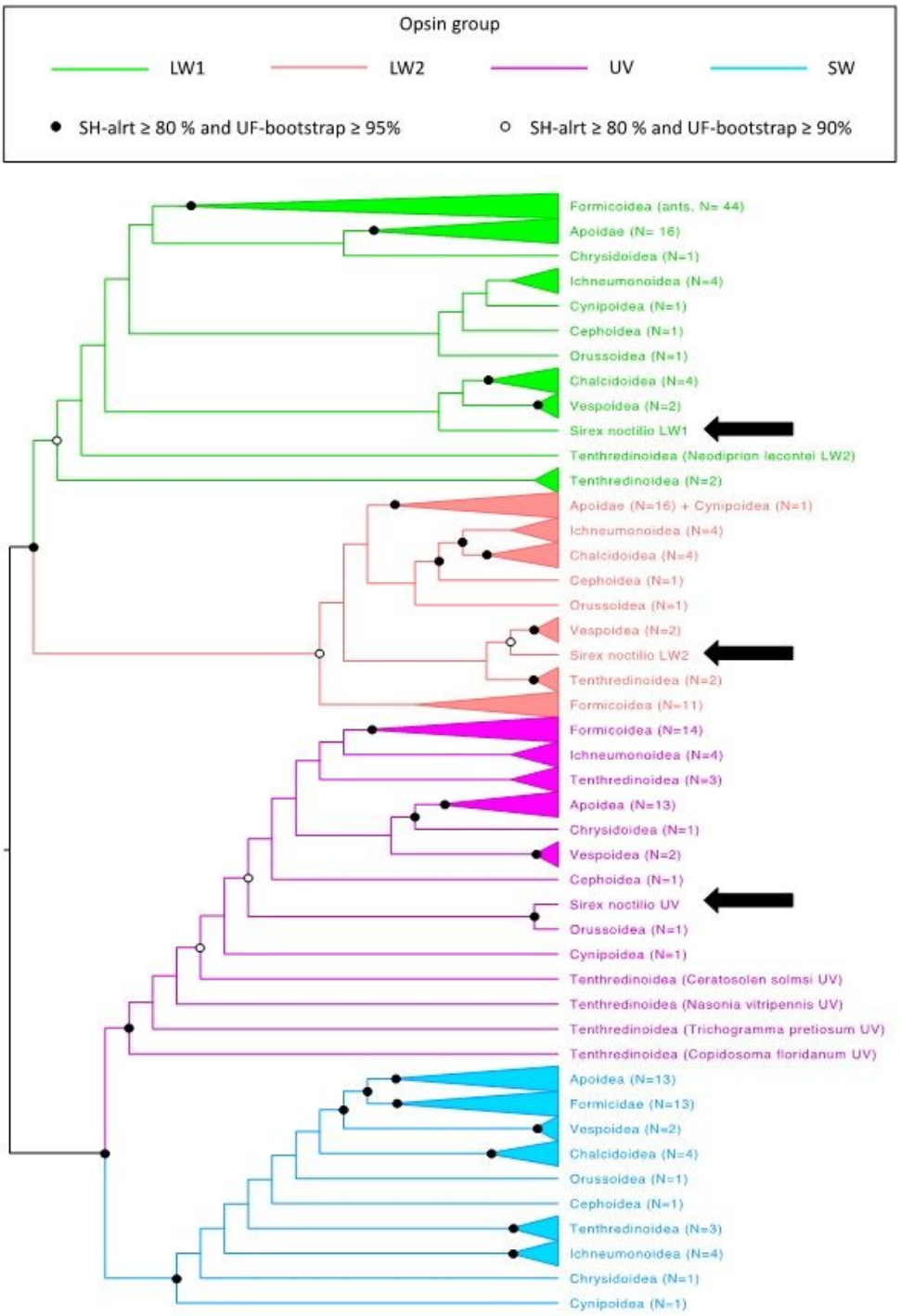

Figure 2

Hymenopteran maximum likelihood tree of LW1 (green), LW2 (red), UV (purple) and SW (blue) opsins. SH-aLRT (out of 10,000 replicates) ≥ 80% and bootstrap value (out of 10,000 replicates) ≥ 90% (●) or ≥ 95% (●) are shown. Position of the visual opsin genes found in S. noctilio were indicated with a black arrow. Accession numbers and curated sequences are available (Table 1, Supplementary material).

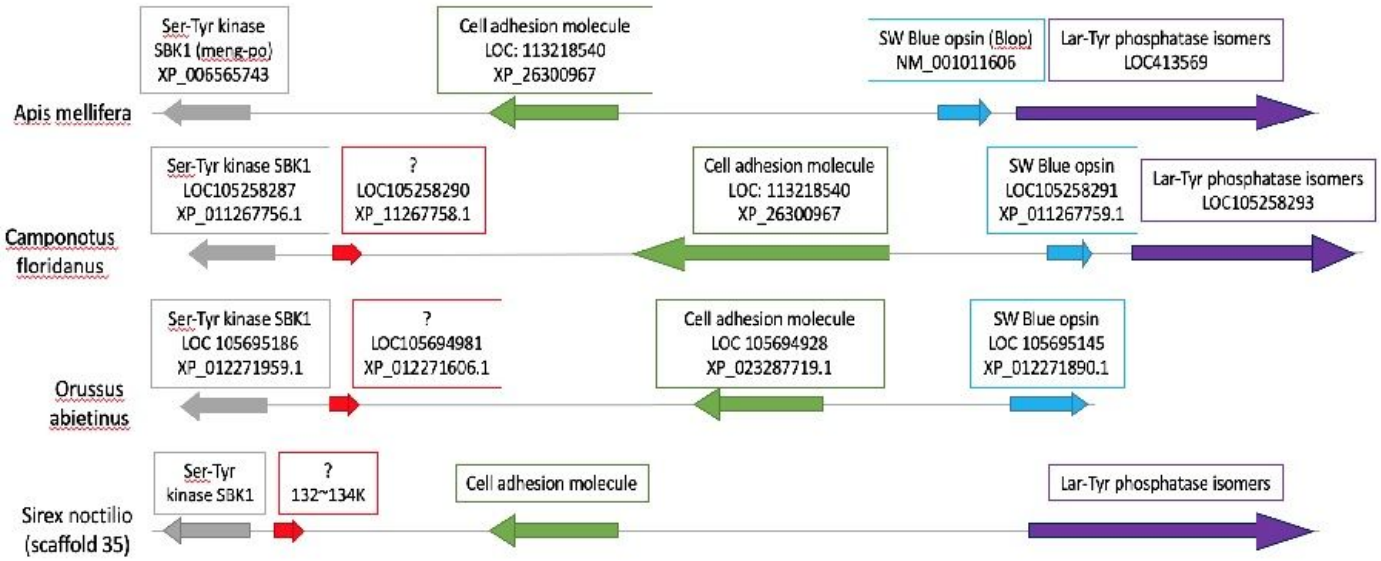

Figure 3

Representation of the SW flanking gene in three hymenopterans. Similar colour corresponds to similar genes. The SW opsin gene in A. mellifera, O. abietinus and C. floridanus is flanked by the Cell adhesion molecule (green) gene and the Lar-Tyr phosphatase (purple) genes, also found in S. noctilio.

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