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Cardinalfishes (Apogonidae) show visual system adaptations typical of nocturnally and diurnally active fish

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Abstract

Animal visual systems adapt to environmental light on various timescales. In scotopic conditions, evolutionary time-scale adaptations include spectral tuning to a narrower light spectrum, loss (or inactivation) of visual genes, and pure-rod or rod-dominated retinas. Some fishes inhabiting shallow coral reefs may show activity during the day and at night. It is unclear whether these fishes show adaptations typical of exclusively nocturnal or deep-sea fishes, or of diurnally active shallow-water species. Here, we investigated visual pigment diversity in cardinalfishes (Apogonidae). Most cardinalfishes are nocturnal foragers, yet they aggregate in multispecies groups in and around coral heads during the day, engaging in social and predator avoidance behaviours. We sequenced retinal transcriptomes of 28 species found on the Great Barrier Reef, assessed the diversity of expressed opsin genes and predicted the spectral sensitivities of resulting photopigments using sequence information. Predictions were combined with microspectrophotometry (MSP) measurements in seven cardinalfish species. Retinal opsin expression was rod opsin (*RH1*) dominated (>87%), suggesting the importance of scotopic vision. However, all species retained expression of multiple cone opsins also, presumably for colour vision. We found five distinct quantitative expression patterns among cardinalfishes, ranging from short-wavelength-shifted to long-wavelength-shifted. These results indicate that cardinalfishes are both well adapted to dim-light conditions and have retained a sophisticated colour vision sense. Other reef fish families also show both nocturnal and diurnal activity while most are strictly one or the other. It will be interesting to compare these behavioural differences across different phylogenetic groups using the criteria and methods developed here.

KEYWORDS
comparative biology, fish, molecular neuroscience, sensory system evolution, visual ecology

1 | INTRODUCTION

Colour vision is mediated by comparing sensory inputs of distinct spectral classes of photoreceptors defined by their morphology and the part of the wavelength spectrum to which each is maximally sensitive (λ_{max}). The outer segments of photoreceptors contain two molecular building blocks of photopigments: the chromophore (often a Vitamin-A derived, light sensitive molecule), and an opsin (a transmembrane protein) to which the chromophore is covalently bound (Hauser & Chang, 2017; Yokoyama, 2008). Vertebrate visual

opsins are divided into five classes dependent on their evolutionary history, photoreceptor specificity and the light spectrum range to which they tune the photopigment (Hunt & Collin, 2014). Rhodopsin (RH1) is found in rods that are used in dim light vision. SWS1 (UV), SWS2 (violet-blue), RH2 (blue-green) and LWS (yellow/red) are found in cones and facilitate colour vision (Yokoyama, 2000). Over evolutionary and shorter timescales, the repertoire of visual opsins can adapt to the light environment inhabited by a species and/or other ecological demands via gene deletion, gene duplication, gene conversion and differential gene expression. Furthermore, pigment sensitivities may be tuned via changes to the amino acid sequence of the opsins due to single nucleotide polymorphisms (SNPs) (reviewed in Carleton, 2009; Hauser & Chang, 2017; Yokoyama, 2008).

Many diurnal animals have a broad palette of opsin genes which facilitate colour vision and associated behavioural tasks. On the other hand, in nocturnal animals and those that live in dim photic environments, such as the deep-sea or caves, a subset of the original opsin genes have been lost or are not expressed at a functional level. For example, nocturnal owls have lost their violet/UV sensitive opsin (*SWS1*), which is found in diurnal raptors, presumably because of the low incidence of short-wavelength light at night. Both the blue-sensitive (*SWS2*) and red-sensitive opsins (*LWS*) experienced strong positive selection early on during owl evolution, which may be linked to the shift from diurnal to crepuscular hunting in their ancestor (Wu et al., 2016). In cetaceans, inactivated copies of *SWS1* are common and several cetacean lineages that dive to depths of over 100 m have convergently lost their *LWS* opsin. Since mammals lost their *SWS2* and *RH2* opsins ancestrally, presumably as a consequence of an evolutionarily prolonged nocturnal phase (Gerkema, Davies, Foster, Menaker, & Hut, 2013; Heesy & Hall, 2010), these cetaceans are effectively rod monochromats, despite exposure to broad spectrum light during breathing intervals at the sea surface (Meredith, Gatesy, Emerling, York, & Springer, 2013; Schweikert, Fasick, & Grace, 2016).

The visual systems of fishes range from pure-rod retinas containing only RH1 in some deep-sea species (Davies et al., 2009; de Busserolles et al., 2015; Hunt, Dulai, Partridge, Cottrill, & Bowmaker, 2001; Musilova et al., 2019; Partridge, Archer, & Vanoostrum, 1992), to highly diversified duplex retinas containing both RH1 and multiple different cone opsins in many shallow-water fishes, facilitating colour vision in these broad spectrum waters (Carleton, 2009; Hofmann et al., 2012; Lythgoe, 1979; Marques et al., 2017; Matsumoto, Fukamachi, Mitani, & Kawamura, 2006). The opsin gene repertoire and their expression is primarily related to the different ecological demands of a species. In freshwater cichlids, for example, it is thought that the plastic expression of opsin genes in response to changes in the environment has facilitated adaptive radiation (Carleton, Parry, Bowmaker, Hunt, & Seehausen, 2005; Kocher, 2004; Seehausen, 2006; Seehausen et al., 2008; Terai et al., 2006). The predominant ecological factor driving spectral sensitivity adaptation is the light environment (Bowmaker et al., 1994; Hauser & Chang, 2017; Lythgoe, Muntz, Partridge, Shand, & Williams, 1994). For example, in deep-sea fishes, the lack of UV and red light in their environment has resulted in a loss of the *LWS* and *SWS1* opsins, whereas their *RH1* opsin is tuned to shorter wavelengths than in shallow-water fishes (Musilova et al., 2019). In diurnal, shallow-water species, only a subset of the various cone opsin genes present in their genomes are expressed at any one time (Carleton & Kocher, 2001), and among several fish clades, levels of expressed opsins differ depending on the spectral regimes that different species inhabit (Carleton, Dalton, Escobar-Camacho, & Nandamuri, 2016; Dalton, Lu, Leips, Cronin, & Carleton, 2015; Fuller & Claricoates, 2011; Fuller, Noa, & Strellner, 2010; Johnson, Stanis, & Fuller, 2013; Sakai, Ohtsuki, Kasagi, Kawamura, & Kawata, 2016; Shand et al., 2008). Finally, colour vision may also be strongly influenced by behavioural tasks such as mate choice, foraging, and predator avoidance (Pegram & Rutowski, 2014; Sandkam, Young, & Breden, 2015; Seehausen et al., 2008; Stieb et al., 2017).

The diversity of spectral sensitivities among coral reef fish, living in one of the most colourful ecosystems on earth, is yet to be explained and probably has no simple answer (for review see Marshall, Cortesi, de Busserolles, Siebeck, & Cheney, 2018). Part of this diversity is known to be facilitated by differential opsin gene expression and amino acid substitutions at opsin gene tuning sites, e.g., in damselfishes (Pomacentridae) (Hofmann et al., 2012; Stieb, Carleton, Cortesi, Marshall, & Salzburger, 2016; Stieb et al., 2017), wrasses (Labridae) (Phillips, Carleton, & Marshall, 2016), and dottybacks (Pseudochromidae) (Cortesi, Feeney, et al., 2015; Cortesi et al., 2016). However, it is unclear how the light environment and/or behavioural tasks drive these processes. There are some clear trends and correlations with increasing depth or water turbidity on and around reefs (Lythgoe, 1979; Lythgoe et al., 1994) and even a loose relationship between fish colours and spectral sensitivities (Marshall, Jennings, McFarland, Loew, & Losey, 2003). None of these factors seem to account for the diversity between reef fish genera and more comparative work is needed, especially with specific behavioural tasks in mind (Losey et al., 2003; Marshall et al., 2003).

Here, we investigated the visual system evolution, in terms of opsin gene diversity and gene expression, in cardinalfishes (Apogonidae). Cardinalfishes are small, abundant, and highly diverse, mainly carnivorous fishes that live in shallow reef habitats (Allen, Steene, Humann, & Deloach, 2003; Barnett, Bellwood, & Hoey, 2006; Helfman, 1986; Marnane & Bellwood, 2002). Most species are nocturnal foragers, while social behaviours are carried out during the day (Brandl & Bellwood, 2014; Kuwamura, 1983, 1985; Marnane & Bellwood, 2002; Saravanan et al., 2013). Independent of activity period, they are frequently preyed on by large predatory fish (Kingsford, 1992). Despite considerable activity during the day, their visual systems, at the anatomical level, show typical dim-light adaptions including a high rod-to-cone ratio (Fishelson, Ayalon, Zverdling, & Holzman, 2004). Therefore, we hypothesized that: (a) cardinalfishes would have rod opsin dominated retinas; (b) their *RH1* opsin would be under positive selection, and (c) they would express a less diverse repertoire of cone opsin genes compared to exclusively diurnal species.

To address these hypotheses, we investigated visual pigment diversity and relative expression from retinal transcriptomes in 28 cardinalfish species found on the Great Barrier Reef (GBR), Australia. We also conducted microspectrophotometry (MSP) in seven species, and compared measured peak spectral sensitivities (λ_{max}) with

estimated λ_{max} values based on opsin gene sequences. This allowed us to infer to which extent opsin gene sequence variation and differential opsin gene expression contribute to visual pigment λ_{max} variability in these enigmatic reef fishes.

2 | MATERIALS AND METHODS

2.1 | Study species

Fish were caught on SCUBA using a clove oil solution, and hand and barrier nets from reefs around Lizard Island (14°40′S, 145°27′E) at depths between 1 and 6 m. All collections occurred between February 2015 and March 2017, and fish were collected under the following permits: Great Barrier Reef Marine Park Authority (GBRMPA) Permit (G12/35005.1), GBRMPA Limited Impact Permit (UQ006/2014) and Queensland General Fisheries Permit (140763). Additional fish were obtained from an aquarium supplier (Cairns Marine Pty Ltd). Animals used for RNA studies were anaesthetized using a clove oil solution (10% clove oil, 40% ethanol, 50% seawater) and sacrificed by decapitation between 12 and 4 p.m. Retinas were removed from the eyecup by dissection and immediately put in RNAlater (ThermoFisher). Tissues were stored at −20°C until transferred to −80°C upon returning to the University of Queensland, Brisbane, Queensland. The molecular phylogenetic relationships of our study species were reconstructed using the methods in Mabuchi, Fraser, Song, Azuma, and Nishida (2014) (Figure 1; for details see Supporting Information p.2).

2.2 | Opsin gene mining

We sequenced retinal transcriptomes for 1–8 individuals per species (73 individuals/28 species; Table S1 and S2) and identified opsin genes by inferring opsin gene phylogenies with known fish opsin genes (Figure S1). To do this, all retinas were homogenized using a

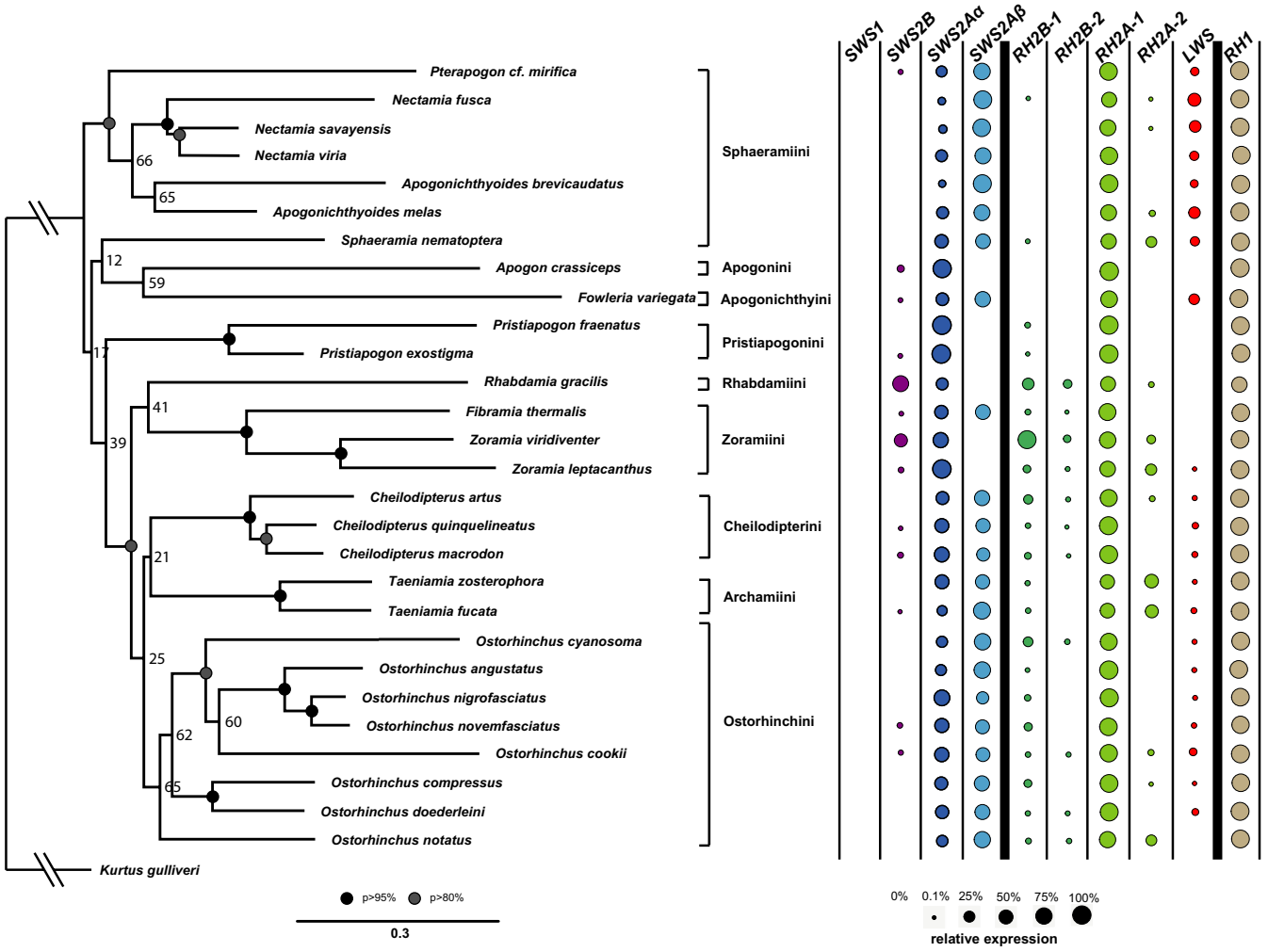

FIGURE 1 Summary of opsin genes expressed in cardinalfishes (Apogonidae) plotted against the cardinalfish phylogeny. Circle diameter represents mean expression level. Missing circles indicate no expression. Expression shown as a fraction of total single cone opsin (*SWS2s*), as a fraction of total double cone opsin (*RH2s*, *LWS*), and as the fraction of rod opsin (*RH1*) versus total opsin expression. Note, cardinalfishes do not express the UV-sensitive *SWS1* gene. Circle colouring is according to the wavelength of maximal sensitivity of opsins according to human vision. Maximum likelihood support values as indicated. SWS = short-wavelength-sensitive; RH1 = rhodopsin 1; RH2 = rhodopsin-like 2; LWS = long-wavelength-sensitive [Colour figure can be viewed at wileyonlinelibrary.com]

tissue lyser LT (Qiagen) and total RNA was extracted using RNeasy Mini Kits (Qiagen) following the manufacturer's protocol. DNAse digestion was performed to eliminate traces of genomic DNA. RNA was quality checked using an Agilent 2100 BioAnalyzer 6000 NanoChip (Agilent Technologies). RNAseq libraries were made in-house by the Queensland Brain Institute's sequencing facility using the TruSeq RNA Sample Preparation Kit v.2 (Illumina), and the retina specific transcriptomes were sequenced as 125 bp paired-end reads on the Illumina platform (HiSeq2000) using SBS chemistry v.4. Samples were multiplexed at 12 samples per lane obtaining between 10 and 30 million sequenced paired-end fragments per sample.

Data was processed using the online Bioinformatics platform Galaxy (Research Computing Centre, The University of Queensland) (Afgan et al., 2015). Reads were quality checked using Trimmomatic (Galaxy v.0.32.2) (Bolger, Lohse, & Usadel, 2014) with the following settings: HEADCROP = 10; SLIDINGWINDOW = 4/20; LEADING = 20; TRAILING = 20; MINLEN = 80. Trinity (Galaxy v.0.0.2) (Grabherr et al., 2011) was used for de novo assembly of transcripts using these settings: group pair distance = 250 bp; minimum inchworm kmer coverage = 2.

Further bioinformatics analyses were performed using Geneious (v.9.0.4). For each species and all opsin genes identified, we followed the methods described in de Busserolles et al. (2017) to manually check for gene duplications. To identify *SWS2A* opsin genes, the assembled transcripts were also mapped to reference opsin gene sequences from the Dusky dottyback (*Pseudochromis fuscus*; GenBank accession No.: KP004335.1). Briefly, after identification of candidate gene coding sequences, unassembled reads were mapped to the opsin gene repertoire of the species using medium-sensitivity settings (70% identity threshold). Allowing for 30% difference between reference and unmapped reads ensures that all reads belonging to a specific copy and its paralogs are mapped, while minimizing unspecific read mapping. Deviating reads were then extracted by working from SNP to SNP by exploiting paired-end matching to cover gaps, and their consensus sequence was used as species-specific reference for repeated high-specificity (100% identity) mapping of unassembled reads until maximum obtainable sequence length was reached.

To confirm the assignment of the newly identified opsin genes to known opsin classes, we aligned their amino acid sequences with the opsin genes of other species, including the zebrafish (*Danio rerio*), Japanese rice fish (*Oryzias latipes*), Bluefin killifish (*Lucania goodei*), a Lake Malawi cichlid (*Metriaclima zebra*), and Nile tilapia (*Oreochromis niloticus*) (Figure S1 for a full list and GenBank accession numbers) using MAFFT (Katoh, Misawa, Kuma, & Miyata, 2002). We then estimated maximum likelihood phylogenies for each gene based on the amino acid sequences using RAXML 8.2.10 (LG+G) (Stamatakis, 2014) on the web based platform CIPRES (Miller, Pfeiffer, & Schwartz, 2010), followed by a rapid bootstrap analysis with 1,000 replicates. The highest scoring tree was selected as the best.

2.3 | Quantitative opsin gene expression

For quantitative opsin gene expression, we assumed *SWS* genes to be expressed exclusively in single cones and *RH2*/*LWS* genes in double cones, based on evidence from several fish species (Dalton, de Busserolles, Marshall, & Carleton, 2017; Dalton, Loew, Cronin, & Carleton, 2014; Dalton et al., 2015; Takechi & Kawamura, 2005; Stieb & de Busserolles, personal communication). Gene expression was then determined according to methods outlined in de Busserolles et al. (2017). In short, unassembled reads of each sequenced specimen were mapped to the coding sequences of identified opsin genes in the respective species using customized high-specificity settings (98% identity, 80 bp minimum read overlap). This ensured specific read-mapping of highly similar sequences while allowing variability due to heterozygous positions. Relative gene expression was then calculated according to:

$$\frac{T_i}{T_{\text{all}}} = \frac{\left(\frac{N_i}{L_i}\right)}{\sum\left(\frac{N_{i+n}}{L_{i+n}}\right)}$$

where T_i/T_{all} is the ratio of gene expression of gene i to all single cone opsin, all double cone opsin, or total opsin; N_i is the number of reads mapped to gene i; L_i is the length of gene i, and n denominates every gene identified in the respective species' transcriptome. This way, gene expression was calculated individually for all identified paralogs, i.e., for each single cone opsin (*SWS2B*, *SWS2A*α, *SWS2A*β) as a fraction of total single cone opsin expressed, each double cone opsin (*RH2B-1*, *RH2B-2*, *RH2A-1*, *RH2A-2*, *LWS*) as a fraction of total double cone opsin expressed, and for rod opsin (*RH1*) as a fraction of total opsin expressed. For species level analyses, expression levels of all specimens per species were averaged (mean ± *SD*). For tribal-level analysis, median expression of all specimens belonging to a phylogenetic tribe was calculated. Tribal categories were adopted from Mabuchi et al. (2014).

For downstream data analysis, quantitative expression data was arc-sin transformed and values for *RH2B-1*/*RH2B-2* and *RH2A-1*/*RH2A-2* were pooled as *RH2B* and *RH2A*, respectively. We then tested for patterns in gene expression using partitioning-around-medoids (PAM) cluster analysis (Kaufman & Rousseeuw, 1990), and performed principal component analysis (PCA) to identify the variables responsible for the clustering using the princomp function in the FACTOMINER package in R v.3.1.2 (Lê, Josse, & Husson, 2008; R Core Team, 2014). The number of clusters used was determined by performing iterative *k*-means calculation of the within-groups-sum-of-squares (WSS), and by then selecting the number of clusters at greatest change in slope (Figure S2). Species of which less than two specimens had been sequenced were excluded from the analysis (*Apogonichthyoides brevicaudatus*, *Ostrorhinchus angustatus*, *Pterapogon* cf. *mirifica*, *Cheilodipterus macrodon*, *Pristiapogon fraenatus*, *Nectamia viria*).

Phylogenetic signal (Pagel's-λ) of mean relative opsin expression per species was calculated using the cardinalfish phylogeny reconstructed here and the phylogenetic least squares (PGLS) function in the CAPER package in R v. 3.1.2 (Orme, 2013).

2.4 | Opsin gene sequence diversification

For amino acid diversity analysis, opsin gene sequences were aligned with bovine rhodopsin (GenBank Accession No.: NP_001014890.1) to allow inference of positions of known tuning sites based on the alignments in Carleton, Spady and Cote (2005) (Tables S3–S8). We focused on potentially functional substitutions, that is substitutions between amino acids that differ in their physical properties (polar, nonpolar, basic, acidic), and that located in the transmembrane region, or substitutions at sites that we identified to be like tuning sites in cardinalfishes but are not yet described. Amino acid sequence diversity was then calculated for each gene by counting the numbers of all variable and all functionally variable sites, all variable and all functionally variable sites in transmembrane regions, all variable and all functionally variable binding pocket sites, as well as functionally variable sites at known tuning sites for the respective opsin gene class and for any opsin gene class as described in Fasick and Robsinson (1998), Yokoyama (2008), and Dungan Kosyakov and Chang (2016). Mean substitution number per gene was calculated to account for different numbers of taxa used due to different availability of complete coding sequences. Sequence diversity was not calculated for *SWS2B*, *RH2A-2* and *RH2B-2* as these genes could not be assembled to complete coding sequences in most species. Nucleotide diversity (π) was calculated in MEGA7 (Kumar, Stecher, & Tamura, 2016). The number of synonymous substitutions per synonymous site (d*S*) and nonsynonymous substitutions per nonsynonymous site (d*N*), were calculated using the codeml program in the software package phylogenetic analysis by maximum likelihood (PAML) on gene specific nucleotide alignments, the amino acid based maximum-likelihood gene trees (Figure S1), and the M0 selection model (Xu & Yang, 2013; Yang, 2007).

Codeml was also used to perform tests for site-specific positive selection following methods explained in detail in Hofmann et al. (2012). Briefly, we performed likelihood ratio tests (LRT) on comparisons of models M1a (neutral) versus M2 (selection), and M8a (neutral) versus M8 (selection). Lastly, we used Bayes empirical Bayes (Yang, Wong, & Nielsen, 2005) to determine sites under positive selection. Due to incomplete opsin coding sequences, we omitted: *Ostorhinchus notatus* and *O. compressus* from the *LWS* alignment; *A. brevicaudatus*, *N. viria*, *Cheilodipterus artus*, *O. notatus*, *Sphaeramia nematoptera*, *P. mirifica*, and *Apogonichthyoides melas* from the *SWS2A*α alignment; *C. macrodon* from the *SWS2A*β alignment; and *C. macrodon*, *O. angustatus*, *O. nigrofasciatus*, *Pristiapogon exostigma*, *P. fraenatus*, *Zoramia viridiventer*, *S. nematoptera*, and *Cheilodipterus quinquelineatus* from the *RH2B-1* alignment.

2.5 | Microspectrophotometry

Microspectrophotometric (MSP) measurements were conducted on seven species: *Zoramia leptacanthus*, *Z. viridiventer*, *Rhabdamia gracilis*, *Ostorhinchus cyanosoma*, *O. cookii*, *O. doederleini*, and *Nectamia savayensis*. Except for *N. savayensis*, fish were wild caught, and housed in aquaria exposed to natural light for brief periods before being transported to the University of Queensland (UQ). *N. savayensis* specimens were acquired through an aquarium supplier (Cairns Marine Pty Ltd). Fish were housed in aquaria at the University of Queensland for no more than one week before measurements. They were kept under a 12 hr/12 hr day/night cycle by fluorescent lighting that included ultraviolet (UV) light. The animals were fed dried marine flakes daily.

Measurements were conducted using a single-beam wavelength-scanning microspectrophotometer. Fish were dark adapted for at least 60 min, and were sacrificed and dissected under infrared illumination immediately before measurements. Following the methods of Shand, Hart, Thomas, and Partridge (2002) and Mosk et al. (2007), small pieces of retinal tissue were teased apart and submerged in a drop of PBS containing 10% sucrose on a 60 mm × 32 mm cover slip. The tissue was then covered with a 19 mm^2 cover slip with greased edges to prevent dehydration. Measurements were performed by placing the outer segment of a single photoreceptor in the path of the measuring beam and scanning the wavelength range from 300 to 800 nm and from 800 to 300 nm in 1 nm increments. Before each cell measurement, a dark scan and a baseline scan were performed in a tissue-free area of the preparation. Transmitted light was collected by a photomultiplier module and converted into an electric signal which was being recorded. The presence of actual photopigment was confirmed by recording the same cell's absorbance after white-light bleaching for 30–60 s to probe for photoisomerization. Each measurement was quality checked according to the methods outlined by Levine and MacNichol (1985) and Partridge et al. (1992), and λ_{max} was inferred for each cell passing these criteria by determining the best-fit regression based on the bovine rhodopsin template from Govardovskii, Fyhrquist, Reuter, Kuzmin, and Donner (2000). Average photoreceptor sensitivities were determined by calculating the mean absorbance spectra of closely clustered λ_{max} measurements of morphologically similar photoreceptor types (Table 2).

2.6 | Spectral sensitivity estimation

Amino acid comparisons of cardinalfish opsins to those of other fish species with known λ_{max} were used to infer spectral tuning effects where possible (see Supporting Information for details). Our opsin gene sequences were aligned with those of *M. zebra*, *O. niloticus*, *L. goodei*, *O. latipes*, and *P. fuscus*. Opsin genes found in these species have been studied extensively, including with in vitro opsin protein expression to assess pure protein spectral absorbance, and MSP (Carleton, 2009; Cortesi et al., 2016; Matsumoto et al., 2006). We focused on variable amino acid residues that occurred in areas corresponding to the retinal binding pocket and where substitutions resulted in a change in polarity, and on substitutions at known tuning sites (Dungan et al., 2016; Takahashi & Ebrey, 2003; Yokoyama, 2008). Estimated cardinalfish opsin λ_{max} were calculated from spectral absorbances of the photopigments of *O. niloticus* (Spady et al., 2006), *L. goodei*

(Yokoyama, Takenaka, & Blow, 2007), or *O. latipes* (Matsumoto et al., 2006; RH1) as opsin sequences among percomorph fishes allow more accurate estimates due to lower sequence variability.

3 | RESULTS

3.1 | Opsin gene mining

We identified multiple visual opsin genes, including cone opsins belonging to three of the four cone opsin classes (*SWS2*, *RH2*, *LWS*) and one rod opsin (*RH1*) in the cardinalfish retinal transcriptomes (Figure 2). Only the *SWS1* opsin class was missing with none of the species expressing the UV-sensitive opsin (Figure 1). We identified three gene duplicates of *SWS2* (*SWS2Aα*, *SWS2Aβ*, *SWS2B*) (also see Cortesi, Musilová, et al., 2015), and found evidence for four *RH2* duplicates (*RH2B-1*, *RH2B-2*, *RH2A-1*, *RH2A-2*).

Every species expressed *RH2A-1*, *RH1* and at least one of the three single cone opsin duplicates (*SWS2Aα*, *SWS2Aβ*, or *SWS2B*); many also expressed *LWS*. However, quantitative expression of single cone opsins, *RH2B-1/RH2B-2*, and *LWS* varied greatly between species (Figures 1 and 2). As a consequence of low expression of a subset of these genes in some species, coding sequences for them could not be assembled to full length in several species (Table 1). *SWS2B*, in particular, could only be fully assembled in *R. gracilis* and *Z. viridiventer*, while it was found to be expressed at just detectable levels in several other species across the family (Figure 2). *R. gracilis* and *Z. viridiventer* lacked *LWS* expression entirely (Figure 2). In fact, the only tribes in which *LWS* could be assembled to complete coding sequences in all members were the Sphaeramiini (seven species) and Apogonichthyini (one species). Several species expressed only two cone opsins at levels allowing assembly to complete coding sequences (i.e., *Apogon crassiceps*, *P. exostigma*), whereas for six species the complete coding sequences of six cone opsins could be assembled (*Fibramia thermalis*, *Taeniamia fucata*, *C. macrodon*, *C. quinquelineatus*, *O. cookii*, and *Ostorhinchus novemfasciatus*).

3.2 | Quantitative opsin gene expression

The retinas of all investigated species were rhodopsin dominated, ranging from 64.1% (±10.9) *RH1* expression in *R. gracilis*, to 98.1% (±0.5) *RH1* expression in *A. crassiceps* (Figures 2 and 3; Table S2). Overall, 24 out of the 28 species expressed over 90% *RH1* in their retinas. Most species expressed at least three cone opsins at levels allowing full coding sequence assembly, while a few species (*A. crassiceps*, *P. exostigma*, *P. fraenatus*) expressed only two cone opsins at such levels. *RH2A-1* showed the highest expression among cone opsins in all species (Figure 2).

We found five distinct gene expression patterns in cardinalfishes (Figure 3), and PCA and cluster analysis revealed that single cone opsin and *LWS* expression explain most of the variability observed between these groups (Figure 3 and Table S10). These patterns are categorized as Group (A) comprising two species (*R. gracilis*, *Z. viridiventer*) that are short-wavelength-shifted, characterized by high levels of *SWS2B* and *SWS2Aα* single cone opsin expression, *RH2B-1* or *RH2B-2* as well as *RH2A-1* double cone opsin expression, and no *LWS* expression. Group (B) consisting of three species (*A. crassiceps*, *P. exostigma*, *P. fraenatus*) expressing predominantly only two cone opsins, *SWS2Aβ* presumably in single cones and *RH2A-1* presumably in double cones. Two intermediate groups are characterized by an almost equal expression of the single cone opsins *SWS2Aα* and *SWS2Aβ*, and mainly *RH2A-1* among double cone opsins. These two groups differ in that members of one expressed *RH2B-1* and/or *RH2B-2* (Group C: *Ostorhinchus nigrofasciatus*, *Ostorhinchus novemfasciatus*, *Cheilodipterus artus*, *O. compressus*, *C. quinquelineatus*, *Taeniamia zosterophora*, *T. fucata*, *O. cyanosoma*, *O. notatus*), whereas members of the other group (Group D: *O. cookii*, *O. doederleini*, *S. nematoptera*, *F. thermalis*, *Fowleria variegata*, *A. melas*) expressed intermediate levels of *LWS* (4.4%–22.0%) as well as *RH2A-1* among double cone opsins. Lastly, a long-wavelength-shifted group (Group E: *Nectamia fusca*, *N. savayensis*), comprises species characterized by near exclusive expression of *SWS2Aβ* among single cone opsins, and high levels of *LWS* (>29%) in addition to *RH2A-1 and/or RH2A-2*.

High variability was observed in single cone opsin expression, ranging from *SWS2B* dominated expression (~70%; e.g., *R. gracilis*), to the expression of nearly equal amounts of *SWS2Aα* and *SWS2Aβ* (some *Ostorhinchus* species, *C. quinquelineatus*, *T. zosterophora*), to *SWS2Aβ* dominated expression (~90%; e.g., *N. fusca*, *N. savayensis*). Similarly, the expression of the second double cone opsin varied considerably between species with *LWS*, ranging from no expression (*R. gracilis*, *A. crassiceps*, *P. exostigma*), to 39.6% (±10.8) expression in *N. fusca*. *RH2B-1* expression ranged from no expression (*N. savayensis*, *A. melas*, *A. crassiceps*) to 40.2% (±10.5) expression in *R. gracilis*.

Opsin gene expression patterns in cardinalfishes show a strong phylogenetic signal (Pagel's λ) for all cone opsins except *SWS2B* (likely due to too few species expressing *SWS2B*; Table S10), with λ being significantly different from zero for these genes, and thus indicating that the expression differences are linked to phylogeny.

3.3 | Opsin gene sequence diversification

We found sequence diversity among all opsins expressed in cardinalfishes. Nucleotide diversity (π) was highest in *SWS2Aα* (0.072), followed by *SWS2Aβ* (0.056) and *LWS* (0.055), and comparably low in *RH1* (0.040), *RH2A-1* (0.036) and *RH2B-1* (0.024) (Table 1). In terms of amino acid substitutions potentially functional for spectral sensitivity tuning, RH1 and LWS show, after SWS2Aα, the greatest variability. This is consistent for transmembrane and binding pocket regions, as well as for substitutions at known tuning sites. Among SWS2Aα opsins, we found five amino acid substitutions that entail a change of physical properties at retinal binding pocket sites, followed by four in RH1, three in LWS, two in RH2B-1, and none in RH2A-1. Four of those in SWS2Aα were known tuning sites in SWS2A opsins, two of those in LWS and RH1, and one of those in RH2B-1, were at known tuning sites for the respective opsin classes.

Using PAML, we found that only RH2A-1 (M8/M8a $p = 3.521 \times 10^{-4}$, M1a/M2 $p = 3.659 \times 10^{-4}$) and RH1 (M8/M8a $p = 3.853 \times 10^{-11}$, M1a/M2 $p = 4.354 \times 10^{-12}$) are under positive selection (Table S9). According to Bayes Empirical Bayes, positive selection has been acting on 18 sites in RH1 and 11 sites in RH2A-1 (Table S9). One of those in RH1 is a known tuning site (S299A/A299S), and nine others show potentially functional substitutions in the transmembrane region. None of the sites in RH2A-1 are known tuning sites, but two (A96S, I222T) are potentially functional substitutions in the transmembrane region.

3.4 | Spectral sensitivity estimation and microspectrophotometry

We present a summary of measured (from MSP; Table 2, Figure S3) and estimated λ_{max} for seven species (Figure 4, Table 2). Overall, opsin

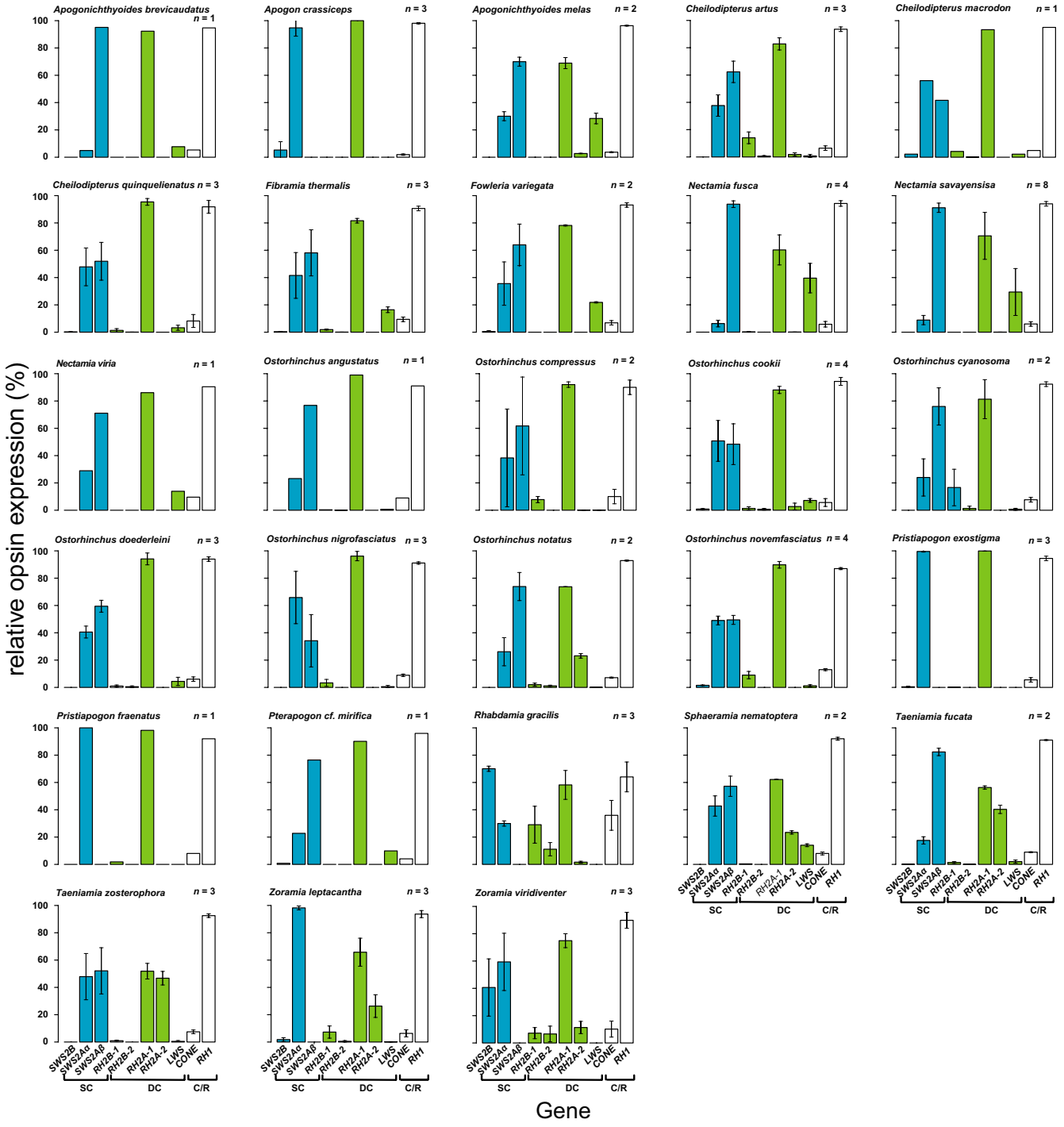

FIGURE 2 Overview of opsin gene expression in cardinalfishes. Relative opsin expression in each species (mean±SD) shown as a fraction of total single cone (SC, blue) opsin (*SWS2s*), as a fraction of total double cone (DC, green) opsin (*RH2s*, *LWS*), and as total cone opsin versus rod opsin (*RH1*) (C/R, white) expression. n = number of specimens used [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Gene diversity among cardinalfish opsins

	SWS2Aα	*SWS2Aβ*	*RH2B-1*	*RH2A-1*	*LWS*	*RH1*
Complete sequences	21	21	13	28	21	28
All sequences	28	23	21	28	23	28
Total number of nucleotides	1,056	1,059	1,038	1,059	1,074	1,065
Variable nucleotide (NT) sites	268	258	115	199	260	193
Mean	12.76	12.29	8.85	7.11	12.38	6.89
Total number of amino acids	352	352	346	352	357	354
Variable amino acid sites	76	50	22	27	54	46
Mean	3.619	2.381	1.692	0.964	2.571	1.643
Functionally variable amino acid sites	26	17	3	6	19	18
Mean	1.238	0.81	0.231	0.214	0.905	0.643
Variable transmembrane sites	46	27	17	19	34	35
Mean	2.191	1.286	1.308	0.679	1.62	1.25
Functionally variable transmembrane sites	16	7	3	2	11	14
Mean	0.762	0.333	0.231	0.071	0.524	0.5
Variable binding pocket sites	10	0	2	0	6	6
Mean	0.476	0	0.154	0	0.286	0.214
Functionally variable binding pocket sites	5	0	2	0	3	4
Mean	0.238	0	0.154	0	0.143	0.143
Functionally variable sites at known or estimated tuning site	4	0	1	0	2	2
Functionally variable sites known to tune other opsin	1	0	3	0	0	0
Nucleotide diversity (π)	0.072	0.056	0.024	0.036	0.055	0.040
Synonymous substitutions (dS) (m0)	1.564	1.558	0.433	1.142	1.764	1.062
Nonsynonymous substitutions (dN) (m0)	0.192	0.138	0.044	0.156	0.182	0.219
dN/dS (m0)	0.123	0.089	0.101	0.136	0.103	0.206

Note: Nucleotide and amino acid sequence diversity for *SWS2B*, *RH2B-2* and *RH2A-2* were not calculated due to low number of fully assembled coding sequences. Species omitted due to incomplete coding sequences: *SWS2Aα* (*A. brevicaudatus*, *A. melas*, *P.* cf. *mirifica*, *S. nematoptera*, *O. notatus*, *C. artus*, *N. viria*), *SWS2Aβ* (*A. crassiceps*, *Z. viridiventer*, *P. exostigma*, *P. fraenatus*, *R. gracilis*, *C. macrodon*, *Z. leptacantha*), *RH2B-1* (*Z. viridiventer*, *A. brevicaudatus*, *A. melas*, *C. macrodon*, *C. quinqelineatus*, *O. nigrofasciatus*, *O. angustatus*, *N. savayensis*, *P. exostigma*, *P. fraenatus*, *S. nematoptera*, *P.* cf. *mirifica*, *A. crassiceps*, *N. viria*, *F. variegata*), *RH2A-1* (none), *LWS* (*Z. viridiventer*, *O. compressus*, *A. crassiceps*, *R. gracilis*, *P. exostigma*, *P. fraenatus*, *O. notatus*), *RH1* (none).

sequences were similar to fish species with known λ_{max} suggesting our estimates are reasonable. Estimated λ_{max} for each cardinalfish opsin class showed little variability as follows. RH1 photopigments were maximally sensitive at 494–502 nm. RH2A-1 was maximally sensitive at 518 nm in all species, with RH2A-2 (where present) maximally sensitive at 522 nm. LWS was maximally sensitive at 544–551 nm. SWS2Aα and SWS2Aβ were maximally sensitive to 444–454 nm, and 468 nm, respectively. RH2B-1 was maximally sensitive at 476 nm in all species except in *N. fusca* (491 nm) (Figure 4). This species shows the substitution T118V, known to blue-shift λ_{max} in RH1 (Takahashi & Ebrey, 2003). However, at the two sites preceding 118, *N. fusca* substituted nonpolar amino acids for polar amino acids (M116R, A117R), which probably strongly red-shifts λ_{max}. RH2B-1 differed from RH2B-2 at only one of the sites thought important for RH2B tuning (F203Y, blue-shifting λ_{max} by 1 nm), thus these pigments are estimated to be maximally sensitive at 480 and 479 nm, respectively (Figure 4).

Microspectrophotometry revealed the presence of one spectral type of rod photoreceptor, at least two types of medium-wavelength-sensitive (MWS) double cone photoreceptors, one (except for *O. cookii*, which had two) short-wavelength-sensitive single cone photoreceptor types (SWS), and two types of medium/long-wavelength-sensitive (M/LWS) double cone photoreceptors (Table 2, Figure S3). Spectral sensitivities varied between taxa, and particularly among SWS and M/LWS cone photoreceptors. Rod cell spectral sensitivities showed little variation, ranging from 497 ± 2 nm in *R. gracilis* to 505 ± 2 nm in *N. savayensis*. We found one double cone with one member maximally sensitive at 500 ± 5 nm (*R. gracilis*) to 507 ± 3 nm (*N. savayensis*), and the other member maximally sensitive at 516 ± 3 nm (*O. doederleini*) to 521 ± 1 nm (*Z. viridiventer*). Furthermore, we found additional double cones sensitive to shorter wavelengths in *R. gracilis* (485 nm) and *N. savayensis* (493 nm), or longer wavelengths (M/LWS) in *O. doederleini* (527 ± 3 nm) and *N. savayensis* (537 ± 2 nm). Single cone spectral sensitivities λ_{max} were the most variable across species: 420 ± 1 nm in *R. gracilis*; 441 ± 5 nm in *Z. viridiventer*; 442 ± 2 nm in *Z. leptacanthus*; 452 nm in *O. cyanosoma*; 453 nm in *O. cookii*; 466 ± 3 nm in *N. savayensis*; 467 nm in *O. doederleini*; 468 nm in *O. cookii*.

TABLE 2 Summary of peak spectral sensitivities (λ_{max}) determined using microspectrophotometry (MSP; SWS single, short-wavelength-sensitive single cone; MWS double, medium-wavelength-sensitive double cone; M/LWS double, medium/long-wavelength-sensitive double cone) and comparison to estimated λ_{max} values, and relative opsin gene expression (mean ± *SD*) as a fraction of total single cone opsin (*SWS2s*) and total double cone opsin (*RH2s*, *LWS*)

MSP λ_{max} (nm)									
Species	**SWS single 1**	**SWS single 2**	**SWS single 3**	**MWS double 1**	**MWS double 2**	**MWS double 3**	**MWS double 4**	**M/LWS double**	**Rod**
Ostorhinchus cookii	–	453 ± 4 *n* = 2	468 ± *n*/a *n* = 1	–	506 ± 1 *n* = 2	519 ± 4 *n* = 17	–		504 ± 4 *n* = 4
Ostorhinchus cyanosoma	–	452 ± *n*/a *n* = 1		–	504 ± 2 *n* = 4	519 ± 4 *n* = 20	–	–	503 ± 2 *n* = 5
Ostorhinchus doederleini	–		467 ± *n*/a *n* = 1	–	502 ± 5 *n* = 8	516 ± 3 *n* = 12	–	527 ± 3 *n* = 8	501 ± 4 *n* = 9
Zoramia viridiventer	–	441 ± 5 *n* = 2	–	–	506 ± 2 *n* = 4	520 ± 4 *n* = 21	–	–	502 ± 2 *n* = 10
Zoramia leptacanthus	–	442 ± 2 *n* = 7	–	–	501 ± 5 *n* = 9	512 ± 2 *n* = 9	521 ± 1 *n* = 8		504 ± 2 *n* = 12
Rhabdamia gracilis	420 ± 1 *n* = 5	–	–	485 ± 4 *n* = 25	500 ± 3 *n* = 11	520 ± 2 *n* = 13	–	–	497 ± 2 *n* = 6
Nectamia savayensis			466 ± 3 *n* = 2	493 ± *n*/a *n* = 1	507 ± 3 *n* = 14	520 ± 3 *n* = 4	–	537 ± 2 *n* = 5	505 ± 2 *n* = 3
Opsin λ_{max} estimates (nm)									
Species	**SWS2B**	**SWS2Aα**	**SWS2Aβ**	**RH2B–1**	**RH2B–2**	**RH2A–1**	**RH2A–2**	**LWS**	**RH1**
Ostorhinchus cookii	–	448	468	476	–	518	522	551	496
Ostorhinchus cyanosoma	–	448	468	476	479	518	522	544	494
Ostorhinchus doederleini	–	448	468	476	479	518	522	551	494
Zoramia viridiventer	407	450	–	480	483	518	–	–	502
Zoramia leptacanthus	407	450	–	476	480	518	522	551	502
Rhabdamia gracilis	420	444	–	476	480	518	522	–	502
Nectamia savayensis	–	456	468	491	–	518	518	544	502
Opsin expression (%)									
Species	***SWS2B***	***SWS2Aα***	***SWS2Aβ***	***RH2B–1***	***RH2B–2***	***RH2A–1***	***RH2A–2***	***LWS***	***RH1***
Ostorhinchus cookii	0.9 ± 0.4	50.8 ± 15.0	48.4 ± 14.9	1.3 ± 1.2	0.7 ± 0.6	88.1 ± 2.6	2.7 ± 2.5	7.2 ± 1.4	–
Ostorhinchus cyanosoma	–	24.0 ± 13.6	76.0 ± 13.6	16.7 ± 13.4	1.3 ± 1.7	81.3 ± 14.3	–	0.7 ± 0.7	–
Ostorhinchus doederleini	–	40.5 ± 4.3	59.5 ± 4.3	1.0 ± 0.8	0.5 ± 0.6	94.1 ± 4.3	0	4.4 ± 3.0	–
Zoramia viridiventer	40.6 ± 21.0	59.4 ± 21.0	–	7.2 ± 4.1	6.6 ± 5.7	74.9 ± 5.1	11.3 ± 4.5	0	–
Zoramia leptacanthus	1.8 ± 1.4	98.2 ± 1.4	–	7.3 ± 4.5	0.5 ± 0.5	65.8 ± 10.3	26.3 ± 8.3	0.1 ± 0.2	–
Rhabdamia gracilis	70.1 ± 1.9	29.9 ± 1.9	–	29.1 ± 13.5	11.1 ± 4.8	58.2 ± 10.6	1.6 ± 0.7	–	–
Nectamia savayensis	–	8.9 ± 3.4	91.1 ± 3.4	–	–	70.5 ± 17.2	0.1 ± 0.0	29.4 ± 17.2	–

n, number of cells scanned.

4 | DISCUSSION

We investigated whether cardinalfishes show adaptations to both dim-light and bright-light vision as a reflection of behavioural activity at night and during the day. We found that cardinalfish opsin gene expression was *RH1* dominated (>87% of total opsin expression; with the exception of *R. gracilis*, ~64%), with much higher expression levels compared to diurnal reef fishes which were sampled at similar times of the day, e.g., damselfishes (Stieb et al., 2017), which typically show expression levels near 60%–70%. This suggests the relative importance of vision in scotopic conditions when most cardinalfish species forage (Barnett et al., 2006; Helfman, 1986; Marnane & Bellwood, 2002). This data also supports anatomical studies, which found high rod-to-cone ratios in the retinas of several members of this family

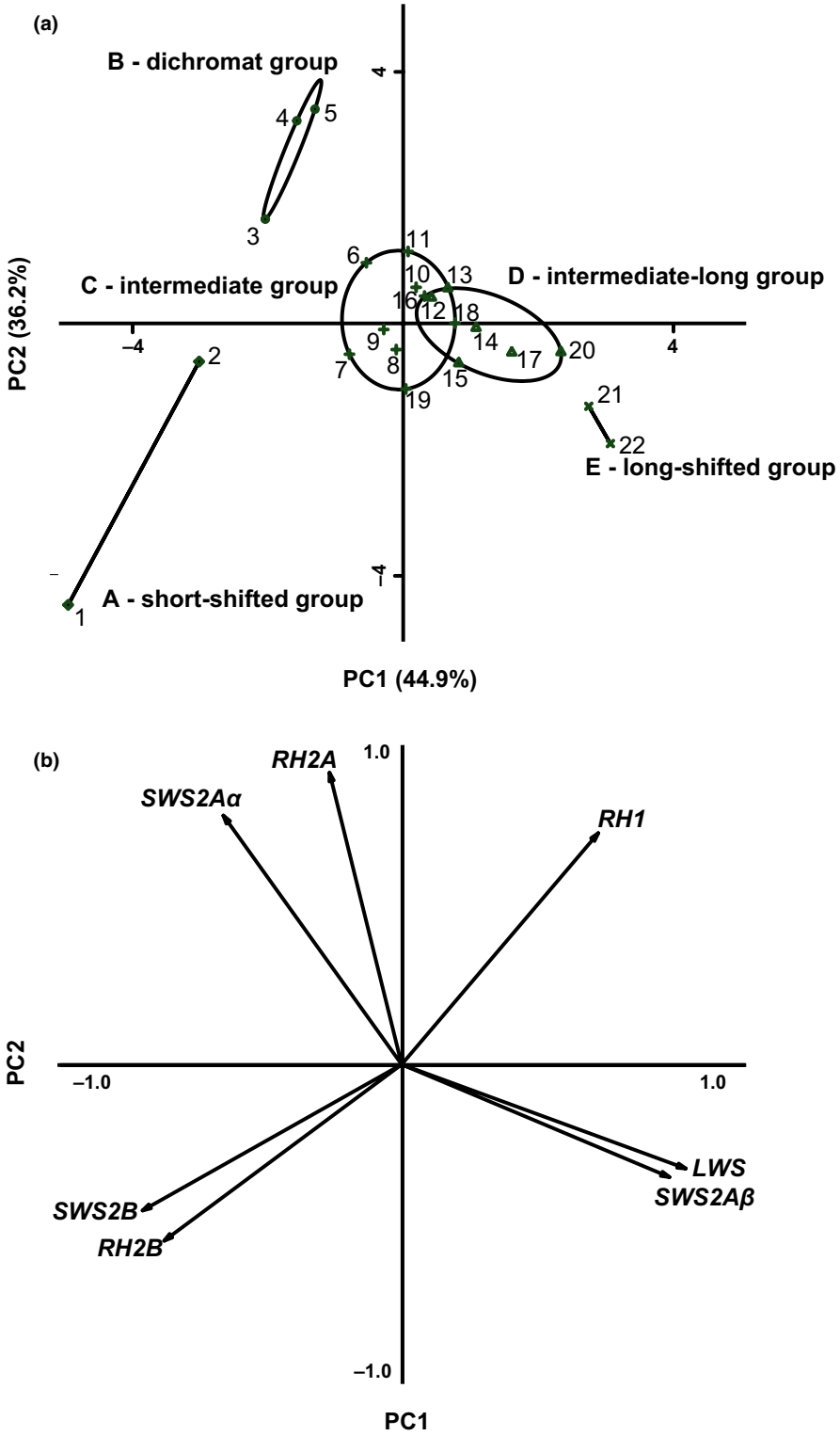

FIGURE 3 Clustering of cardinalfishes by relative opsin expression according to (a) PAM cluster-analysis of relative opsin expression in cardinalfishes. (b) Correlation plot of PCA responsible forclustering. 1 = *Z. viridiventer*, 2 = *R. gracilis*, 3 = *Z. leptacantha*, 4 = *P. exostigma*, 5 = *A. crassiceps*, 6 = *O. nigrofasciatus*, 7 = *O. novemfasciatus*, 8 = *C. artus*, 9 = *O. compressus*, 10 = *C.quinquelineatus*, 11 = *T.zosterophora*, 12 = *O. cookii*, 13 = *O. doederleini*, 14 = *S. nematoptera*, 15 = *F. thermalis*, 16 = *O. notatus*, 17= *F. variegata*, 18 = *T. fucata*, 19 = *O. cyanosoma*, 20 = *A. melas*, 21 = *N. savayensis*, 22 = *N. fusca* [Colour figure can be viewed at wileyonlinelibrary.com]

(Fishelson et al., 2004). However, we also found a high diversity in cone opsin expression between species, which ranged in expression from two to six cone opsin genes, similar in number to diurnal reef fishes (reviewed in Marshall et al., 2018 and Losey et al., 2003 for MSP data).

Species with rod dominated retinas often show a reduction in cone opsin complement (Jacobs, Deegan, Neitz, Crognale, & Neitz,1993; Jacobs, Neitz, & Neitz, 1996; Musilova et al., 2019; Zhao, Rossiter, Teeling, Li, Cotton, & Zhang, 2009). However, the repertoire of expressed cone opsins in the majority of cardinalfish species studied here was at least as diverse as that found in diurnal reef fish species (Cortesi et al., 2016; Phillips et al., 2016; Stieb et al., 2017). In fact, most species expressed three cone opsins and therefore are potentially trichromatic (Cronin, Johnsen, Marshall, & Warrant, 2014). No species expressed *SWS1*, most likely due to this gene having been lost ancestrally (Musilova et al., 2019). Three of our species (*A. crassiceps*, *P. exostigma*, *P. fraenatus*) expressed only two opsins at levels that allowed full coding sequence assembly, and may therefore be dichromats. Our results therefore indicate that, as nocturnal foragers, cardinalfishes are well adapted to dim-light vision, while the ability to perceive colour is maintained. Though cardinalfishes are commonly referred to as nocturnal, most species can be found in large aggregations in light exposed locations during the day, and are known to carry out social behaviours, such as courtship and mating, or predator avoidance, at these times. Whether cardinalfishes may also use colour vision in dim light conditions, as some geckos and anurans do (Kelber & Lind, 2010; Kelber & Roth, 2006; Roth & Kelber, 2004; Yovanovich et al., 2017), requires further exploration.

The diurnal ecological demands of reef fishes could be seen as broadly similar. They all live in the same water type (Jerlov, 1977) over a similar depth range and feed against and in many cases on similarly coloured objects (e.g., coral, algae, turf-algae or sand-substrate). The variation seen in spectral sensitivity must be explained by other factors (Marshall et al., 2018), including environmental or behavioural ones. Finer-scale habitat choice (i.e. microhabitat, such as inside coral or outside coral branches) may determine the variability seen, or alternatively specific food requirements and/or mate choice. Using cluster analysis and PCA on quantitative opsin expression data from cardinalfishes, we identified possible functional groupings within the family that may be indicative of such demands. We found five distinct opsin expression profiles, categorized by the types and amounts of cone opsins expressed along a short-to-long wavelength gradient. Though we do not know what shaped this trend specifically, several aspects in cardinalfish ecology may have contributed to colour vision diversification in this group. Fish visual systems are generally adapted to the overall light available in their environment (Crescitelli, McFall-Ngai, & Horwitz, 1985; Lythgoe, 1979). Therefore, small differences in habitat depth (Stieb et al., 2016; Terai et al., 2017) and substrate (Sabbah et al., 2011) can influence opsin expression according to gradients in available light and environmental reflectance, respectively, leading to diverse photoreceptor spectral sensitivities (Cronin et al., 2014; Lythgoe et al., 1994). Within the boundaries of the available light, colour sensitivity may also be shaped by specific behavioural tasks, such as mate choice, conspecific recognition, or foraging (Marshall, Vorobyev, & Siebeck, 2006; Marshall, 2000; Marshall & Vorobyev, 2003; Miyagi et al., 2012; Sandkam et al., 2015).

Differences in habitat depth are unlikely to explain the cardinalfish groupings as all specimens were collected from 1 to 6 m depth, and therefore should have experienced similar general light environments. Likewise, all fishes were collected from a sheltered lagoon reef environment, arguably providing little diversity in habitat lighting between collection locations. This is unlike the larger scale geographical differences between snapper species (Lutjanidae) whose

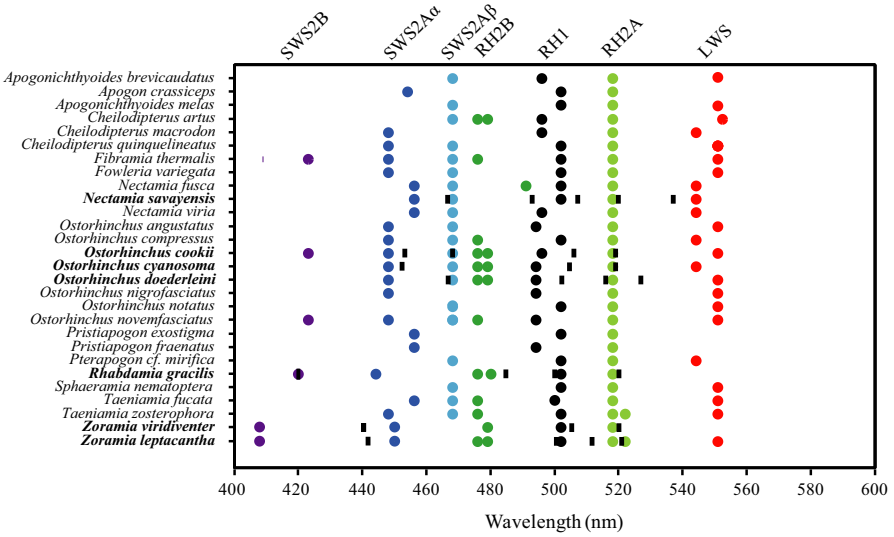

FIGURE 4 Overview of estimated peak spectral sensitivity (λ_{max}) values for cardinalfish opsins. Coloured dots = estimated values; black bars = photoreceptor sensitivities determined via microspectrophotometry (MSP). If sequence information at sites considered important for tuning was missing, no dot is plotted for that opsin. Species with MSP data in bold. Violet = SWS2B; dark-blue = SWS2Aα, bright-blue = SWS2Aβ, dark-green = RH2B-1 (second, longer shifted = RH2B-2, where present); light-green = RH2A-1; red = LWS; black = RH1 [Colour figure can be viewed at wileyonlinelibrary.com]

photoreceptor spectral sensitivity repertoires reflected the different light environments of their marine habitats, from coastal waters to offshore reefs (Lythgoe et al., 1994). However, differences in habitat use and illumination on a much smaller scale (microhabitat), may impact colour vision diversification in fishes (Cummings & Partridge, 2001; Endler, 1993; Leal & Fleishman, 2002; Levine & MacNichol, 1979; Sabbah et al., 2011). Whether such effects helped shape colour vision diversity on coral reefs, has so far only been hypothesized (Marshall et al., 2003) and remains to be tested. Indeed, during the day, cardinalfishes frequently occur in multi-species aggregations where they partition the available space such that some species occupy well sheltered and hidden microhabitats, whereas others are found more exposed (Gardiner, 2010; Gardiner & Jones, 2005).

Cardinalfish opsin expression showed a strong phylogenetic signal and could perhaps be a result of phylogenetically subdivided niche partitioning. However, as it is unknown whether the light available in those microhabitats is at all different and whether such differences relate to the microhabitat use and opsin gene expression profiles of cardinalfishes, further investigation is required to clarify this. Regardless, the repertoire of expressed cone opsins in the short-shifted group (*Z. viridiventer*, *R. gracilis*; Figure 3, Table 2, Table S10), seems well suited for a midwater lifestyle where blue wavelengths dominate (Gardiner, 2010; Kuiter & Tonozuka, 2001; McFarland, 1991). *R. gracilis* in particular, with the highest cone opsin expression of all cardinalfish species, seems well adapted to daylight vision. This species was the most similar in terms of cone opsin expression to other diurnal reef fishes, such as the damselfishes (Stieb et al., 2017), wrasses (Phillips et al., 2016), and dottybacks (Cortesi et al., 2016). Conversely, species occupying sheltered microhabitats, e.g., *Nectamia bandanensis* (Gardiner, 2010), might show adaptations typical of scotopic species. Though *N. bandanensis* was not included in this study, all *Nectamia* species tested here had long-shifted cone opsin repertoires and expression, a characteristic that may be related to longer-shifted light in sheltered coral, crevice or cave microhabitats on coral reefs (Marshall et al., 2003).

In some fishes, body colouration may have coevolved with colour vision as needed in con- and interspecific communication (Hauser & Chang, 2017; Marshall et al., 2003; Marshall & Vorobyev, 2003; Sandkam et al., 2015). Cardinalfishes show diverse body colourations, ranging from near transparent (e.g., *R. gracilis*) to red (e.g., *A. crassiceps*), often combined with intricate stripe patterns (Allen et al., 2003; Randall, Allen, & Steene, 1990). Conspecific and/or interspecific recognition may in fact play a critical role in orchestrating the habitat partitioning behaviour at their diurnal aggregation sites. Different selective pressures may act on different species depending on their own body colouration and that of the species they frequently share, or avoid sharing, a habitat with, thus leading to diversification of the cone opsin gene repertoire and expression. Many cardinalfishes also show ultraviolet facial markings (Marshall et al., 2006; Marshall, 2000; Marshall & Vorobyev, 2003) similar to those observed in damselfishes, which use these markings to identify conspecifics (Partridge & Cuthill, 2010; Siebeck, Parker, Sprenger, Mäthger, & Wallis, 2010). Though indications for a correlative relationship between mate colouration, mate choice and colour vision adaptation are available from various systems (Carleton et al., 2016; Fuller & Noa, 2010; Johnson et al., 2013; Sandkam et al., 2015), this alone seems implausible to explain colour vision diversity observed even among only cardinalfishes let alone all reef fishes. Cardinalfishes do not show colour associated sexual dimorphism, again making it difficult to use this to explain visual system diversity among species (Okuda, Miyazaki, & Yanagisawa, 2002; Okuda, Tayasu, & Yanagisawa, 1998; Okuda & Yanagisawa, 1996; Vagelli, 2011).

Predation efficiency is enhanced in various zooplanktivorous fishes that are sensitive to UV-light (Job & Bellwood, 2007; Job & Shand, 2001; Johnsen & Widder, 2001; Jordan, Howe, Juanes, Stauffer, & Loew, 2004; Leech, Boeing, Cooke, Williamson, & Torres, 2009; Leech & Johnsen, 2006; Losey et al., 1999; Stieb et al., 2017). At the other end of the spectrum, increased expression of *LWS* opsin, and thus an increased sensitivity for long-wavelength light, is thought to enhance foraging efficiency in algivorous fishes due to increased detectability of the long-wavelength chlorophyll signature in algae (Stieb et al., 2017). However, not only are the vast majority of cardinalfish species nocturnal feeders (Barnett et al., 2006; Brandl & Bellwood, 2014; Marnane & Bellwood, 2002), and as such their cone-based colour vision unlikely to be involved in foraging in those species, but cardinalfishes are also almost exclusively carnivorous, and no species tested here is algivorous (Barnett et al., 2006; Frédérich et al., 2017; Marnane & Bellwood, 2002; Vivien, 1975). On the other hand, species showing short-wavelength-shifted opsin repertoires based on *SWS2B* expression (e.g., *Z. viridiventer*, *R. gracilis*), may experience increased foraging success in midwaters rich in short-wavelength light. As *Z. viridiventer* feeds nocturnally, this may be most relevant for *R. gracilis* which feeds during the day (Kuiter & Tonozuka, 2001).

Opsin gene sequence diversity was found to be greatest in *SWS2A*α, *SWS2A*β and *LWS* opsins based on nucleotide diversity (π). However, at the amino acid level, *SWS2A*α, *LWS* and *RH1* showed the greatest variability. This was notable with respect to substitutions occurring at retinal binding pocket or known tuning sites, indicating that these cone opsins may be important in driving the adaptive diversification of cardinalfish colour vision. This is supported by PCA which identified *SWS* and *LWS* opsins as the greatest contributors to interspecific expression differences. These results agree with patterns observed in other teleosts, including other reef fishes (Hofmann et al., 2012; Stieb et al., 2017), as protein sequence diversity is generally greater in the genes coding for short- (*SWS1*, *SWS2*) and long-wavelength-sensitive opsins (*LWS*). This phenomenon may be due to these parts of the light spectrum being the most variable as a result of scatter (short wavelengths), greater attenuation with increasing depth, viewing distance, or organic matter load (long wavelengths) (Jerlov, 1977; Levine & MacNichol, 1982; Lythgoe, 1988), as well as blue-shifting of the light spectrum during twilight (de Busserolles et al., 2017; Lythgoe, 1979) and increased proportions of red-light around sunset (McFarland, 1991; McFarland & Munz, 1975). These effects could be critical for successful predator avoidance during such times of increased predation risk on coral reefs (Danilowicz & Sale, 1999; Helfman, 1986; McFarland & Wahl,

1996). However, since *RH1* and *RH2A-1* were found to be under positive selection, an overall stronger selective pressure on dim-light and luminance vision, that all species in the family share is also likely.

In line with differences in sequence diversity, photoreceptor spectral sensitivity also showed the greatest λ_{max} variation between cardinalfish species in short (SWS single) and medium/long-wavelength-sensitive photoreceptors (M/LWS double) based on MSP measurements and photopigments (SWS, LWS) based on estimates. The overall high similarity in λ_{max} of estimated visual pigments, furthermore suggests that actual differences found *in vivo* are due to differential cone opsin expression and/or the coexpression of opsin genes within the same photoreceptors, rather than sequence diversification per se (Figure 4). However, it is also possible that changes in opsin gene expression in individuals housed in aquaria for a short-time, lead to unnatural shifts in spectral sensitivities. Measured sensitivities matched estimated ones for single cone types 2 (SWS2Aα) and 3 (SWS2Aβ), and double cone type 3 (RH2A-1). For example, in *N. savayensis* we found single cones with a λ_{max} of 466 nm, matching the estimated λ_{max} for SWS2Aβ (468 nm) which is the most expressed single cone opsin in this species (91.1% ± 3.4). However, measurements and estimates did not match for some single and double cone sensitivities in several other species when considering quantitative gene expression. For example, in *Z. viridiventer*, only one type of single cone, with a λ_{max} of 441 nm, was found. Yet, this species expresses predominantly *SWS2Aα*, predicted to be maximally sensitive at 450 nm, and lower levels of *SWS2B*, predicted to be maximally sensitive at 407 nm. In *R. gracilis* we found only one single cone type with a λ_{max} of 420 nm, matching the prediction for SWS2B. Yet, *R. gracilis* also expressed *SWS2Aα* (30%) with a predicted λ_{max} of 444 nm. The λ_{max} measured in *Z. viridiventer* could be achieved by mixing photopigments that use different opsins in individual single cones, a phenomenon shown to occur in cichlids (Dalton et al., 2017). In the case of *R. gracilis*, however, based on gene expression, we would have expected to find either a single cone sensitive to wavelengths between 420 and 444 nm (if genes were coexpressed), or two distinct types of single cones, each maximally sensitive to the above wavelengths. Given the low replicate number for MSP measurements and expression data, it is unclear whether the mismatch is because we missed single cones containing SWS2Aα, whether single cone opsin expression does in fact vary more than our results captured, or whether our estimates are inaccurate.

Our RH2A-1 estimates largely matched the λ_{max} measured for one double cone member in each species. Nevertheless, we also found medium-wavelength-sensitive (MWS double) photoreceptors with a λ_{max} of approximately 500–505 nm in all species, and an M/LWS double cone with a λ_{max} of 537 nm in *N. savayensis*. These cones do not match the predicted λ_{max} of any double cone opsin in any species, but could be explained by opsin gene coexpression; in this case of *RH2B-1* or *RH2B-2* with *RH2A-1*, and *RH2A-1* with *LWS*. This agrees with studies showing coexpression of *RH2A* and *LWS* double cone opsin genes in cichlids (Dalton et al., 2015) and dottybacks (Cortesi et al., 2016), to yield photoreceptors maximally sensitive at intermediate wavelengths. Opsin gene coexpression could explain the double cone with a λ_{max} of 500 nm that was present in all tested species, i.e., those that express both *RH2A* and *RH2B* genes; however, it is not convincing for species that lack *RH2B-1/RH2B-2* expression (e.g., *O. doederleini*, *O. cookii*, *N. savayensis*). The λ_{max} of all long(er)-wavelength-sensitive photoreceptors found were significantly shorter than those estimated for LWS. However, as they were found exclusively in species that showed *LWS* expression, we conclude that the measured sensitivities also result from opsin gene coexpression with *RH2* genes. Although long-wavelength-shifting of fish visual pigments could also be achieved through chromophore substitution, from vitamin A1 to A2, this is unlikely to explain the shifts observed here as marine fish primarily utilize the A1 based 11-cis retinal (Toyama et al., 2008).

5 | CONCLUSION

Our study highlights that even among members of mostly nocturnally active coral reef fishes, the cardinalfishes, which possess rod (*RH1*) dominated visual systems, cone photoreceptor spectral sensitivities are remarkably diverse. The variability is based on an equally diverse repertoire of cone opsin genes. As sequence differentiation between genes was low and *RH2A-1* was the only cone opsin gene under positive selection, differential gene expression accounts for most of the observed variability, and gene coexpression is likely to facilitate fine-scale spectral tuning. Within the cardinalfish family, species show cone opsin expression patterns along a short-to-long wavelength-sensitive-shifted gradient. The implications of this differentiation remain unclear pending further investigation around diet, mate selection, and other behavioural parameters. In particular, a comparative approach may identify ecological factors that have helped shape colour vision among other coral reef fishes. For example, the holocentrid family (squirrelfish and soldierfish) are also mainly nocturnally active for foraging but possess both daytime activities and cones in their eyes (de Busserolles, F., Stieb, S. unpublished data). Whether they possess similar spectral sensitivities to the cardinalfish due to activity pattern or show differences due to divergent diet and phylogenetic background remains one of the interesting questions for the future.

AUTHOR CONTRIBUTIONS

M.L., K.L.Ch. and N.J.M. designed the study; M.L. collected the data; M.L., K.L.Ca., F.C. analysed the data; M.L. wrote the initial manuscript. All authors contributed to writing the manuscript and approved the final version.

DATA ACCESSIBILITY

Newly identified sequences and sequenced transcriptomes are available through GenBank (Figure S1 for accession numbers) and the SRA archive (PRJNA529507). All other data used is available

through Dryad (https://doi.org/10.5061/dryad.t0p2f2c) or is provided in either the main manuscript or the Supporting Information.

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