Genomic evidence of a functional RH2 opsin in New Zealand parrots and implications for pest control

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Abstract

Recent genomic evidence suggest that kea (*Nestor notabilis*) have a non-functional RH2 opsin gene potentially leading to impaired vision in the green region of the electromagnetic spectrum. In New Zealand, it is standard procedure to add green dye to aerial poison baits used in mammalian predator control operations to deter native birds from eating toxic bait. A visual deficiency could impact how kea perceive and interact with green-dyed baits and thus have unforeseen consequences for kea conservation. Here, we sequenced the partial RH2 gene of seven wild kea and re-analysed the kea genome raw sequencing data of the RH2 locus. We demonstrate that the reported premature stop codon is most likely an assembly artefact. An extended analysis of the published genomes of all three extant New Zealand parrots (Superfamily: Strigopoidea) confirms that the RH2 gene is functional in this entire group.

Introduction

Birds are living in a visual world (Walls 1943). Birds rely heavily on their sense of vision for a variety of activities, including foraging (Viitala *et al.* 1995; Tedore and Nilsson 2019), reproduction (Bennett *et al.* 1996) and movement (Wagner and Sauer 2010; Muheim 2011). The bird's retina, like that of other vertebrates, consists of two types of photoreceptors, rod and cone cells, which express different types of photopigments or opsins. Rod cells contain rhodopsin (RH1), responsible for low-light vision. Cone cells express cone opsins, which underlie colour vision. In birds cone opsins can be divided into four subgroups corresponding to their light absorption spectra: a medium-wavelength sensitive opsin (RH2), an opsin sensitive to long wavelengths (LWS), and two types of short-wavelength sensitive opsins (SWS1, SWS2). Thus most birds possess a tetrachromatic visual system (reviewed in Hart 2001 and Hart and Hunt 2007).

Recently, the Bird 10K (B10K) consortium investigated avian opsin genes in 363 species across the bird phylogeny as part of a large comparative genomics project (Feng *et al.* 2020). The consortium found that RH1 and RH2 were present in all birds but were incomplete or pseudogenised in a small number of species (5 and 11 respectively). The remaining three genes showed varied patterns of presence and absence (Feng *et al.* 2020). An earlier comparative genomics study of avian opsins had revealed a pseudogenised RH2 gene in the barn owl (*Tyto alba*) and a segmental deletion within RH2 in penguins, consistent with adaptation to a nocturnal and an aquatic lifestyle in the owl and in penguins, respectively (Borges *et al.* 2015).

The kea (*Nestor notabilis*), a large and endangered parrot endemic to the South Island of New Zealand (Higgins 1999) was one of the species reported in Feng *et al.* (2020) to have a premature stop codon in RH2, potentially leading to impaired vision in the green region of the electromagnetic spectrum (λmax = 499–506 nm; Hart and Hunt 2007). A deficiency in green colour vision could have unforeseen consequences for kea conservation in New Zealand. Introduced mammalian predators such as stoats, rats, and possums have been devastating New Zealand's native biodiversity and one of the most effective eradication tools to protect the unique flora and fauna are large scale aerial drops of sodium fluoroacetate, 1080 poison baits (Towns *et al.* 2013; Russell and Broome 2016). As standard practice, green dye has been added to aerial poison baits to deter native birds from eating toxic bait pellets (Caithness and Williams 1971), based on studies that have shown avoidance of green food items by several avian species (Cowan and Crowell 2017). Aerial poison drops using green toxic bait are frequently preceded by drops of undyed or green-dyed non-toxic pre-feed bait to increase subsequent consumption of toxic baits by neophobic predators. The effects of using either undyed or green pre-feed bait on non-target avian species remains unknown (Cowan and Crowell 2017; Brunton-Martin *et al.* 2021).

77 Kea may be of particular by-kill risk from aerial drops as they are omnivorous ground-feeders,
78 intelligent and inquisitive birds (Diamond and Bond 1999), and readily explore novel food
79 objects (Kemp *et al.* 2019). They may directly feed on poison bait, especially if they have learnt
80 that non-toxic pre-feed bait is an acceptable food source (Orr-Walker and Roberts 2009).
81 Brunton-Martin *et al.* (2021) modelled the appearance of different predator control baits based
82 on "average parrot vision", to gauge the ability of kea to discern bait from different
83 backgrounds and between dyed and undyed bait. The study's findings suggest that kea are
84 likely able to distinguish between green-dyed and undyed baits in well-lit environments and
85 that green-dying baits likely had a camouflage effect (Brunton-Martin *et al.* 2021). The authors
86 highlighted the need to review the current practice of using undyed pre-feed and green toxic
87 baits in the light of their findings but they also acknowledged that their model based on average
88 parrot vision might not reflect the true visual capabilities of kea. In this context, it is essential
89 to further investigate the claim of a non-functional RH2 gene, and its potential implications for
90 green vision impairment in kea.

91 In this study, we sequenced the partial RH2 gene of seven wild kea and re-analysed the B10K
92 kea genome raw sequencing data of the RH2 locus. We demonstrate that the reported premature
93 stop codon is most likely an assembly artefact in the reference genome. Further, we extended
94 our analyses to the published genomes of all three extant New Zealand parrots, kea, kākā
95 (*Nestor meridionalis*), and kākāpō (*Strigops habroptilus*), (superfamily: Strigopoidea) and
96 confirm that the RH2 gene is functional in this entire group.

Material and Methods

Kea, kākā, and kākāpō reference assemblies

No information has been published on the location of the premature stop codon within the coding sequence (CDS) of the RH2 locus in kea (Feng *et al.* 2020). For further examination, we identified and retrieved the RH2 gene region from the kea reference genome assembly ASM69687v1 (GenBank accession: GCA_000696875.1) by running an NCBI blastn search with the zebra finch (*Taeniopygia guttata)* RH2 mRNA as query (accession: NM_001076696.1). We also retrieved the RH2 sequence of the two other New Zealand parrot species, the kākāpō (accession: GCF_004027225.2; Dussex *et al.* 2021) and the kākā (Martini *et al.* 2021) for comparison. A multiple sequence alignment of the RH2 CDS for kea, kākā, kākāpō, and zebra finch was generated using the web application of MAFFT v. 7 (Katoh *et al.* 2019) with default parameters.

Reassembly of the RH2 locus

The RH2 genes in the kea and kākā reference assemblies contained some unresolved sequence (N-stretches), therefore we performed a reassembly of the RH2 gene region for both species. Specifically, we mapped the kea raw sequencing reads (SRR959225 - 27) against the scaffold of the reference genome containing the RH2 gene (NW_009924444.1; 24,924 bp) using the bwa mem algorithm from BWA v. 0.7 (Li 2013). Similarly, the kākā raw reads (Martini *et al.* 2021) were mapped to scaffold ps_chr26 (4.90 Mbp) of the kākā reference assembly. Using Samtools view v1.13 (Li *et al.* 2009), we extracted all mapped reads (-F 4) from a 10 kb and a 100 kb region surrounding the RH2 locus on the kea and kākā scaffolds, respectively. We converted the mapped reads from bam to fastq format with BamUtil's bam2FastQ v. 1.0.14 (Jun *et al.* 2015). Adapters and low quality bases ($q < 10$) were trimmed with TrimGalore v.

0.6.4 (Krueger *et al.* 2021). Reads shorter than 40 bp after trimming were discarded. Trimmed reads were then assembled into scaffolds with ABySS v. 2.0.2 (Jackman *et al.* 2017) with k-mer size set to 64. We used Geneious Prime v. 2020.2.2 (Biomatters Ltd.) to visualise and manually curate the resulting sequences. Scripts used for the reassembly are available on Github (https://github.com/StefanieGrosser/Kea_RH2opsin).

Primer design

We used the Primer3Plus web application (Untergasser *et al.* 2007) to design PCR primers targeting the region containing the presumed premature stop codon. We designed the primers in sequence regions conserved between the three New Zealand parrot species to allow cross-amplification: the forward primer in exon 3 (Kea_RH2_Exon3_578CDS_F: 5'-CCCACAACCCTGACTATCACA-3') and reverse primer in exon 4 (Kea_RH2_Exon4_840CDS_R: 5'-TCCCTTGTTGGTGAAGATCC-3').

Kea DNA extraction, PCR, and Sanger sequencing

DNA was extracted from seven kea blood samples held at the Department of Zoology, University of Otago for conservation related studies (Wildlife Act permit nr. 78375-DOA) using a standard phenol-chloroform extraction protocol (Sambrook and Russell 2001). PCR reactions were set up in 25 µl volumes containing 1× PCR buffer, 1.5mM $MgCl_2$, 200 µM of each dNTP, 0.5 U of Taq DNA polymerase (BioTaq, Bioline USA Inc.), and 0.5 µM of each primer. The thermocycling conditions were an initial denaturation of 2 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 50°C for 40 sec and 72°C for 1 min; followed by a final extension of 10 min at 72°C. PCR products were purified using Acroprep 96 filter plates (Pall Corporation) following the manufacturer's protocol. Sanger sequencing of PCR products in

both directions was performed on an ABI3730xl at the Genetic Analysis Services, Otago University, Dunedin New Zealand.

Sanger sequence analysis

Sanger sequences were edited in Geneious Prime. Primer sequences and low quality 5'-ends were manually trimmed from the sequences. Forward and reverse sequences were aligned using the pairwise Geneious Alignment option with default parameters and a consensus sequence was generated (no mismatches between forward and reverse sequences allowed). A multiple alignment including the full CDS of RH2 extracted from the kea reference genome and the partial CDSs extracted for the seven representative kea samples was generated using the web application of MAFFT v. 7 with default parameters.

Results

We extracted and aligned the full CDS of the RH2 opsin gene from the zebra finch, kea, kākā, and kākāpō reference genomes (1,068 bp in length, 356 amino acid residues). We identified the premature stop codon previously reported for kea at the beginning of exon 4 at residue 239 (a glutamic acid in all other sequences) caused by a single G⇒T nucleotide substitution at the first codon position (Figure 1A &1B). Additionally, we observed an amino acid change from alanine to glutamine (residue 233) caused by two nucleotide substitutions at the first and second codon position. Moreover, the full gene sequence revealed that intron 3 of the kea RH2 gene contained a stretch of unknown sequence (Ns) close to the start of exon 4. Because of the close proximity of the 2 non-synonymous substitutions in exon 4 (causing a loss of function of the gene), the N-stretch in intron 3, as well as the gene's position at the scaffold edge, we suspected that the kea reference assembly might be of low quality in this region of the genome and the premature stop might represent an assembly artefact rather than a true loss of function mutation.

We attempted to verify this hypothesis in two ways. We Sanger sequenced this particular gene region in seven individual wild kea. We found that none of these samples had a premature stop codon at residue 239, and instead all sequences contained a glutamic acid at this position (Figure 1C). Similarly, at residue 233 none of the samples had the two nucleotide substitutions causing the alanine to glutamine change in the reference assembly. Finally, Sanger sequencing resolved the 31 bp N stretch at the 3'-end of intron 3 as two missing base pairs—an A and a C —in the reference assembly. We also identified four polymorphic sites within the alignment of the 7 wild kea (Supplementary Figure S1); three sites in exon 3 and one site in exon 4. Interestingly, the polymorphism identified at position 674 of the CDS nucleotide alignment, a G⇒A transition at the 2nd codon position of residue 225, results in a non-conservative amino acid replacement (arginine to histidine). This polymorphism was found in only one individual wild kea, which was heterozygous at this site (the polymorphism was confirmed from the forward and the reverse sequencing read).

To further assess the possibility of an assembly artifact in the kea reference genome at the RH2 locus, we reassembled this genomic region from the raw reads. We extracted and reassembled 8,025 reads that mapped to scaffold NW_009924444.1 (corresponding to a mean scaffold coverage of approx. 32X). This resulted in a new assembly of 37 scaffolds with an N50 of 3,046 bp (range: 72 - 5651 bp). We identified scaffolds containing the RH2 gene by using NCBI blastn against the nr/nt database and a subsequent manual alignment of positive hits against the kea RH2 gene in Geneious Prime. Only seven scaffolds (range: 145 - 545 bp) aligned to the RH2 gene (covering ~68% of the gene). The shortest scaffold of 145 bp aligned within exon 4 and contained a glutamic acid at residue 239 (premature stop codon in the reference). Residue 233 was not contained within this short scaffold (or any of the other scaffolds). Additionally, we examined the bam file (NW_009924444.1 with mapped raw reads)

189 using IGV v. 2.8.2 (Robinson *et al.* 2011). Residue 239 was covered by 11 reads of which nine
190 supported a G instead of a T.

191 Similar to the kea, we reassembled the RH2 locus for kākā by mapping raw reads against a
192 100kb scaffold region of the reference assembly. We assembled a 15,493 bp scaffold
193 containing the RH2 locus. While this scaffold also contained several N stretches within intronic
194 regions, we could successfully resolve the first 29 bp of the missing nucleotide sequence in
195 exon 2 (which is identical to the kea, Supplementary Figure S2). Overall, we observed 14
196 conservative and 2 non-conservative amino acid substitutions between the zebra finch and the
197 New Zealand parrots, and four conservative amino acid replacements between kākāpō and the
198 two *Nestor* species (for three of these changes kea and kākā showed the same amino acid
199 identity as the zebra finch).

200 **Discussion**

201 In this study we examined if the kea RH2 opsin gene contains a premature stop codon as
202 previously reported by Feng *et al.* (2020), which would be indicative of a non-functional green
203 opsin gene. We Sanger sequenced several wild kea individuals at the RH2 gene region
204 containing the presumed stop codon and reassembled the genomic region from raw sequencing
205 data of the kea genome assembly. We show that kea have an intact RH2 gene and suggest that
206 the published kea genome (GCA_000696875.1) is likely misassembled at this locus.

207 The kea genome assembly was generated as part of a comparative avian genomics study which
208 included the first 48 available bird genomes (Zhang *et al.* 2014). The genome is assembled
209 from low coverage (32X) Illumina short-read data with two insert-size libraries and is highly
210 fragmented. Missing, truncated, or incorrectly assembled genes are common in such short-read
211 assemblies (Yin *et al.* 2019; Rhie *et al.* 2021). While the loss or pseudogenisation of RH2 has

been established in several avian (Borges *et al.* 2015; Le Duc *et al.* 2015; Wu *et al.* 2016) and other vertebrate lineages (Bowmaker 2008), our results show that the loss of function previously reported for kea RH2 (Feng *et al.* 2020) is an artifact caused by low genome assembly quality. Our reassembly of the RH2 locus from raw sequencing data resulted in many short contigs and did not allow for the reconstruction of the entire gene, however, a short fragment matching parts of exon 4 showed the expected glutamic acid at position 239 where the kea reference assembly contains the premature stop codon. Our results may suggest that functional loss of the RH2 (and other opsin) genes in other avian species reported in Feng *et al.* (2020) could equally originate from assembly artefacts. More generally, evolutionary inference based on comparative genomics studies that rely on highly fragmented or low-quality genome assemblies warrants careful assessment.

Despite providing evidence for a functional RH2 gene, our analyses alone cannot determine the true visual capabilities of kea and the other two New Zealand parrots. The models employed by Brunton-Martin *et al.* 2021 based on "average parrot vision" seem to remain a reasonable proxy for kea vision. We concur with (Brunton-Martin *et al.* 2021) that more research is required to determine keas' behaviour towards green-dyed and undyed types of baits used in aerial predator control operations.

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Data availability statement

RH2 opsin sequences for wild kea have been deposited in NCBI GenBank under accessions XXXXX (for individuals sharing identical sequences only one representative sequence has been deposited). Access to kākā genome data is subject to iwi consultation. For details please see Martini *et al.* 2021.

Disclosure statement

The authors have no conflicts of interest to declare.

Authors contributions

BCR, AF, SG, YF, LD conceived the study. BCR provided samples, FR performed lab work. SG and LD performed sequence analysis. MK and DM provided sequence data for kaka. SG, with the help of LD, YF, and BCR wrote the manuscript. All authors contributed to the final version of the manuscript.

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Figures

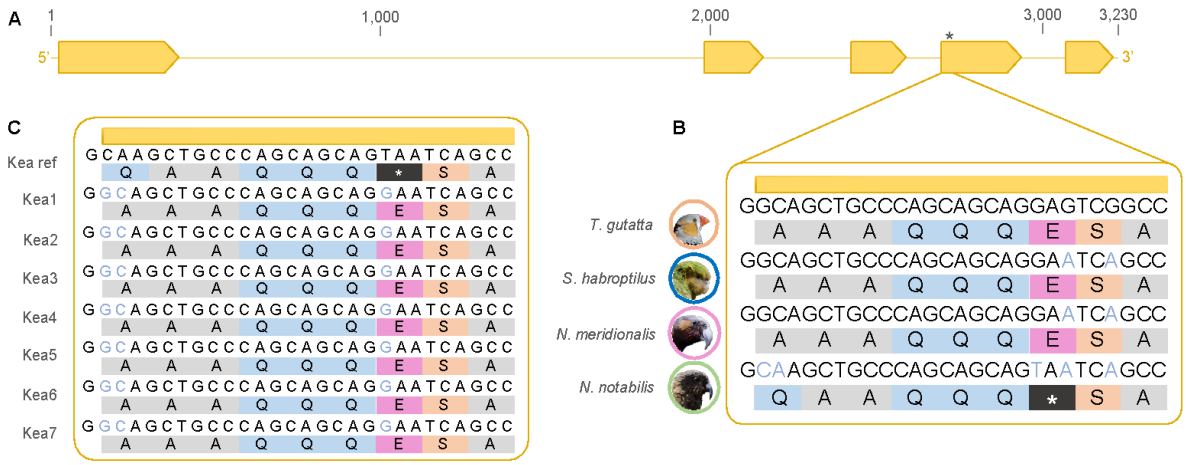

Figure 1. Avian RH2 opsin gene. **A** Schematic of the zebra finch RH2 gene (GenBank accession NC_045024: LOC751972) with exons depicted as arrows. The asterisk indicates the position of the premature stop codon in the kea reference assembly. **B** Nucleotide sequences and corresponding amino acid translations for the zebra finch, kākāpō, kākā, and kea shown for the 5'-end of exon 4. Nucleotide substitutions in the New Zealand parrots compared to the zebra finch are shown in light blue. **C** Nucleotide sequences and corresponding amino acid translations for the kea reference assembly and seven kea samples ascertained with Sanger sequencing for the 5'-end of exon 4. Nucleotide substitutions in the kea samples compared to the reference assembly are shown in light blue.

Supplementary Figures

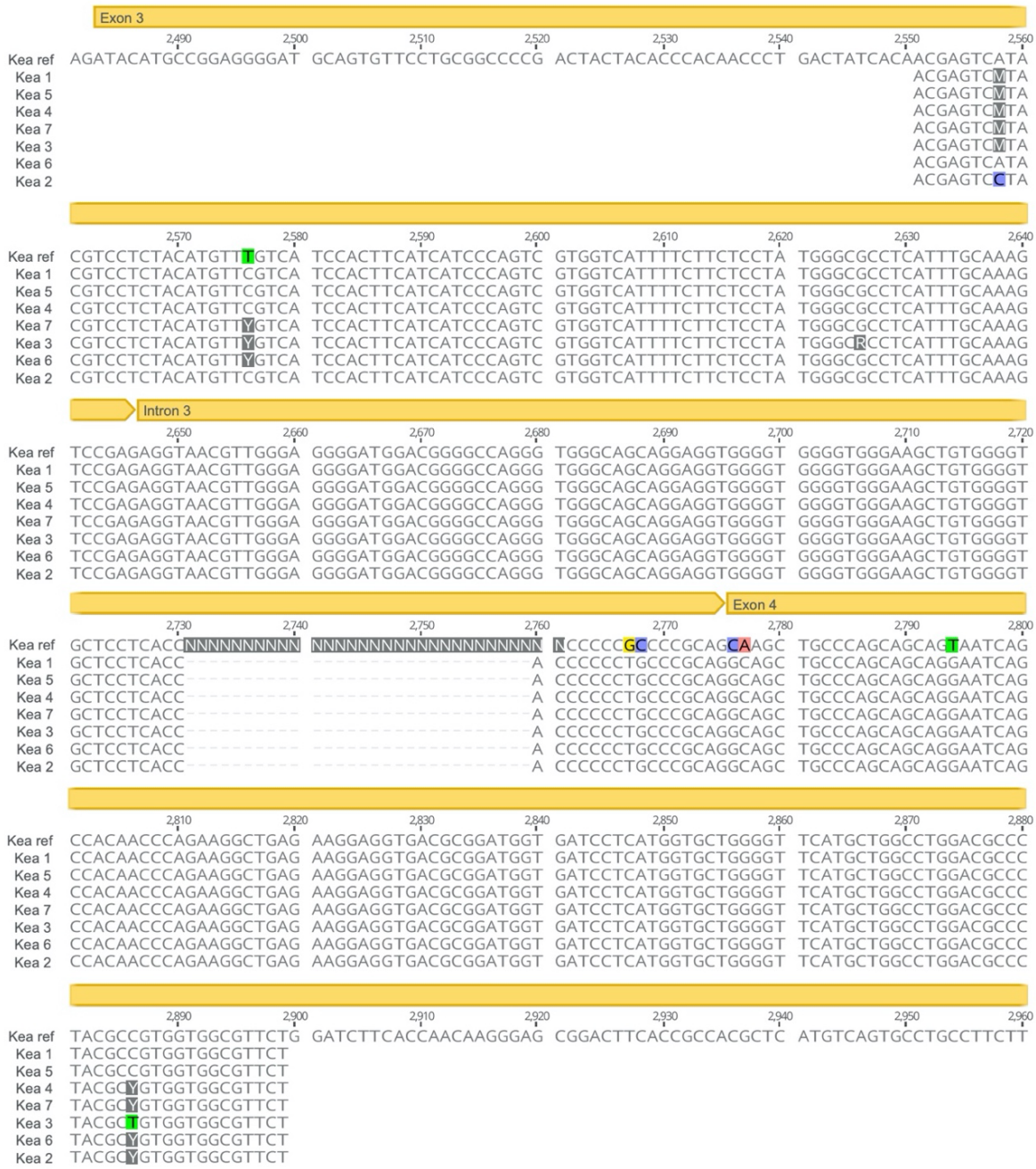

Figure S1. Partial nucleotide sequences alignment of the RH2 gene for the kea reference assembly and seven kea samples ascertained with Sanger sequencing (exon 3, intron 3 and partial exon 4). The yellow bar above the sequence indicates the start and stop position of the exons and intron. Coloured nucleotides and amino acids highlight polymorphisms between the different samples.

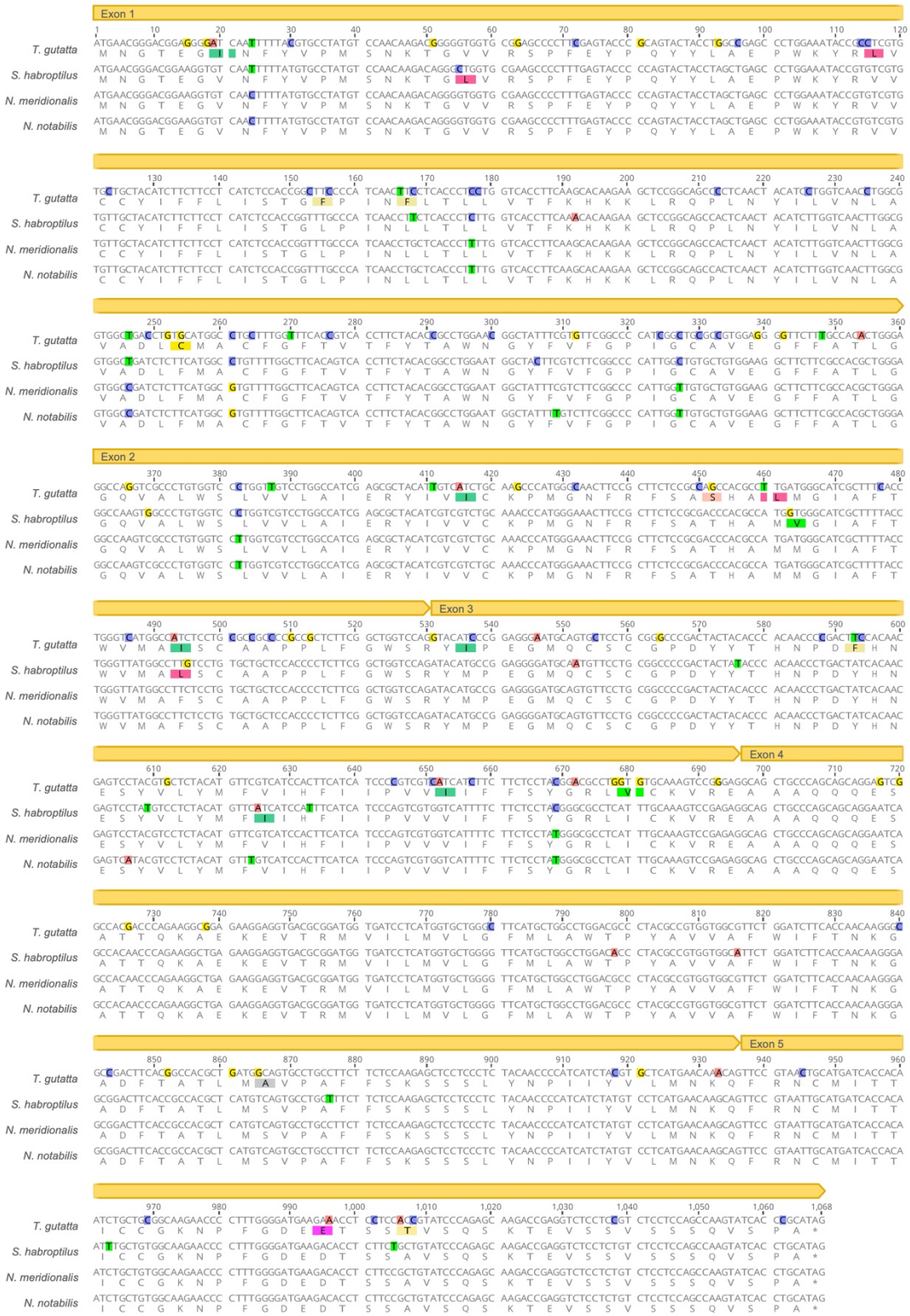

Figure S2. RH2 gene CDS nucleotide alignment (and amino acid translation) for zebra finch, kākāpō, kākā, and kea (the later sequences have been corrected based on the results of this study). The yellow bar above the sequence indicates the start and stop position of the exons. Coloured nucleotides and amino acids highlight polymorphisms between the different species.