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

3 Stefanie Grosser[a], Ludovic Dutoit[a], Yasmin Foster[a], Fiona Robertson[a], Andrew E. Fidler[b],
4 Denise Martini[c], Michael Knapp[c,d] and Bruce C. Robertson[a]

5 *[a]Department of Zoology, University of Otago, Dunedin, New Zealand; [b]Kea Conservation*
6 *Trust, Queenstown, New Zealand; [c]Department of Anatomy, University of Otago, Dunedin,*
7 *New Zealand; [d] Coastal People, Southern Skies Centre of Research Excellence, Department of*
8 *Anatomy, University of Otago, Dunedin, New Zealand*

9 Correspondence:

10 Stefanie Grosser, Email: stefanie.grosser[at]otago.ac.nz, Department of Zoology, University of
11 Otago, Dunedin, New Zealand

12 Bruce Robertson, Email: bruce.robertson[at]otago.ac.nz, Department of Zoology, University
13 of Otago, Dunedin, New Zealand

14

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17 ORCID IDs:
18 Stefanie Grosser: https://orcid.org/0000-0002-1524-7255
19 Ludovic Dutoit: https://orcid.org/0000-0002-0164-9878
20 Yasmin Foster: https://orcid.org/0000-0002-0377-1244
21 Andrew Fidler: https://orcid.org/0000-0003-2992-658X
22 Michael Knapp: https://orcid.org/0000-0002-0937-5664
23 Denise Martini:https://orcid.org/0000-0001-8676-9328
24 Bruce Robertson: https://orcid.org/0000-0002-5348-2731
25

26 **Abstract**

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

37 **Introduction**

38 Birds are living in a visual world (Walls 1943). Birds rely heavily on their sense of vision for
39 a variety of activities, including foraging (Viitala *et al.* 1995; Tedore and Nilsson 2019),
40 reproduction (Bennett *et al.* 1996) and movement (Wagner and Sauer 2010; Muheim 2011).
41 The bird's retina, like that of other vertebrates, consists of two types of photoreceptors, rod and
42 cone cells, which express different types of photopigments or opsins. Rod cells contain
43 rhodopsin (RH1), responsible for low-light vision. Cone cells express cone opsins, which
44 underlie colour vision. In birds cone opsins can be divided into four subgroups corresponding
45 to their light absorption spectra: a medium-wavelength sensitive opsin (RH2), an opsin
46 sensitive to long wavelengths (LWS), and two types of short-wavelength sensitive opsins
47 (SWS1, SWS2). Thus most birds possess a tetrachromatic visual system (reviewed in Hart
48 2001 and Hart and Hunt 2007).

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

57 The kea (*Nestor notabilis*), a large and endangered parrot endemic to the South Island of New
58 Zealand (Higgins 1999) was one of the species reported in Feng *et al.* (2020) to have a
59 premature stop codon in RH2, potentially leading to impaired vision in the green region of the
60 electromagnetic spectrum (λmax = 499–506 nm; Hart and Hunt 2007). A deficiency in green
61 colour vision could have unforeseen consequences for kea conservation in New Zealand.
62 Introduced mammalian predators such as stoats, rats, and possums have been devastating New
63 Zealand's native biodiversity and one of the most effective eradication tools to protect the
64 unique flora and fauna are large scale aerial drops of sodium fluoroacetate, 1080 poison baits
65 (Towns *et al.* 2013; Russell and Broome 2016). As standard practice, green dye has been added
66 to aerial poison baits to deter native birds from eating toxic bait pellets (Caithness and Williams
67 1971), based on studies that have shown avoidance of green food items by several avian species
68 (Cowan and Crowell 2017). Aerial poison drops using green toxic bait are frequently preceded
69 by drops of undyed or green-dyed non-toxic pre-feed bait to increase subsequent consumption
70 of toxic baits by neophobic predators. The effects of using either undyed or green pre-feed bait
71 on non-target avian species remains unknown (Cowan and Crowell 2017; Brunton-Martin *et*
72 *al.* 2021).

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

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

116 0.6.4 (Krueger *et al.* 2021). Reads shorter than 40 bp after trimming were discarded. Trimmed
117 reads were then assembled into scaffolds with ABySS v. 2.0.2 (Jackman *et al.* 2017) with k-
118 mer size set to 64. We used Geneious Prime v. 2020.2.2 (Biomatters Ltd.) to visualise and
119 manually curate the resulting sequences. Scripts used for the reassembly are available on
120 Github (https://github.com/StefanieGrosser/Kea_RH2opsin).

121 *Primer design*

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

128 *Kea DNA extraction, PCR, and Sanger sequencing*

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

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

140 *Sanger sequence analysis*

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

148 **Results**

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

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

174 To further assess the possibility of an assembly artifact in the kea reference genome at the RH2
175 locus, we reassembled this genomic region from the raw reads. We extracted and reassembled
176 8,025 reads that mapped to scaffold NW_009924444.1 (corresponding to a mean scaffold
177 coverage of approx. 32X). This resulted in a new assembly of 37 scaffolds with an N50 of
178 3,046 bp (range: 72 - 5651 bp). We identified scaffolds containing the RH2 gene by using
179 NCBI blastn against the nr/nt database and a subsequent manual alignment of positive hits
180 against the kea RH2 gene in Geneious Prime. Only seven scaffolds (range: 145 - 545 bp)
181 aligned to the RH2 gene (covering ~68% of the gene). The shortest scaffold of 145 bp aligned
182 within exon 4 and contained a glutamic acid at residue 239 (premature stop codon in the
183 reference). Residue 233 was not contained within this short scaffold (or any of the other
184 scaffolds). Additionally, we examined the bam file (NW_009924444.1 with mapped raw reads)

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

187 Similar to the kea, we reassembled the RH2 locus for kākā by mapping raw reads against a
188 100kb scaffold region of the reference assembly. We assembled a 15,493 bp scaffold
189 containing the RH2 locus. While this scaffold also contained several N stretches within intronic
190 regions, we could successfully resolve the first 29 bp of the missing nucleotide sequence in
191 exon 2 (which is identical to the kea, Supplementary Figure S2). Overall, we observed 14
192 conservative and 2 non-conservative amino acid substitutions between the zebra finch and the
193 New Zealand parrots, and four conservative amino acid replacements between kākāpō and the
194 two *Nestor* species (for three of these changes kea and kākā showed the same amino acid
195 identity as the zebra finch).

196 **Discussion**

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

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

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

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

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235 Innovation & Employment's Research Infrastructure programme.

236 **Data availability statement**

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

241 **Disclosure statement**

242 The authors have no conflicts of interest to declare.

243 **Authors contributions**

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

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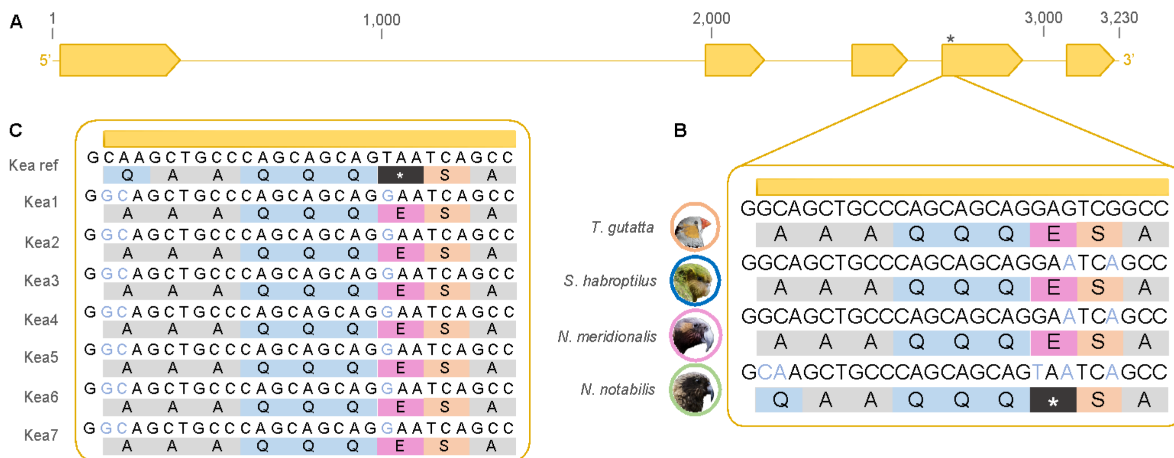
406 **Figures**



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

417

Supplementary Figures

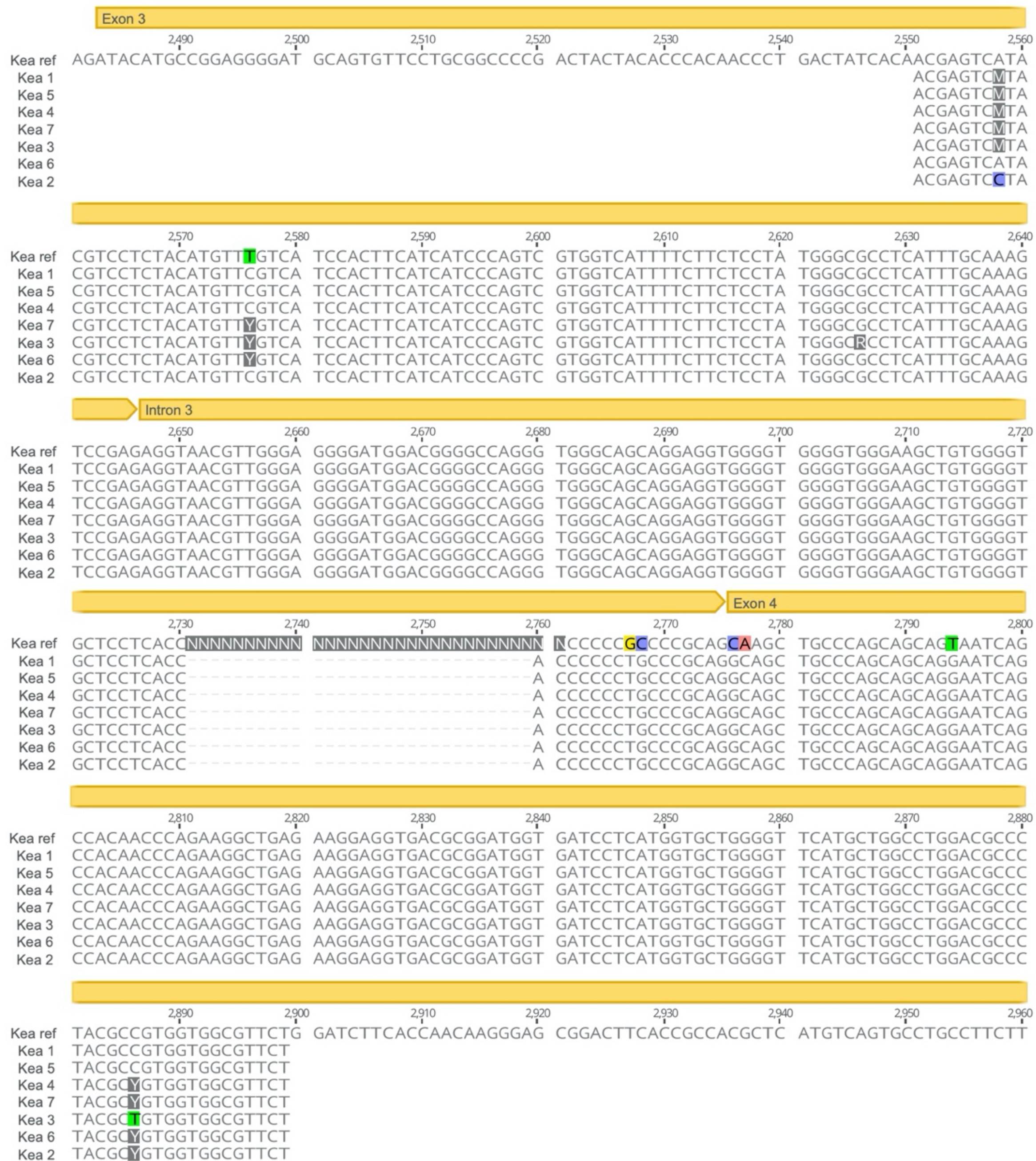
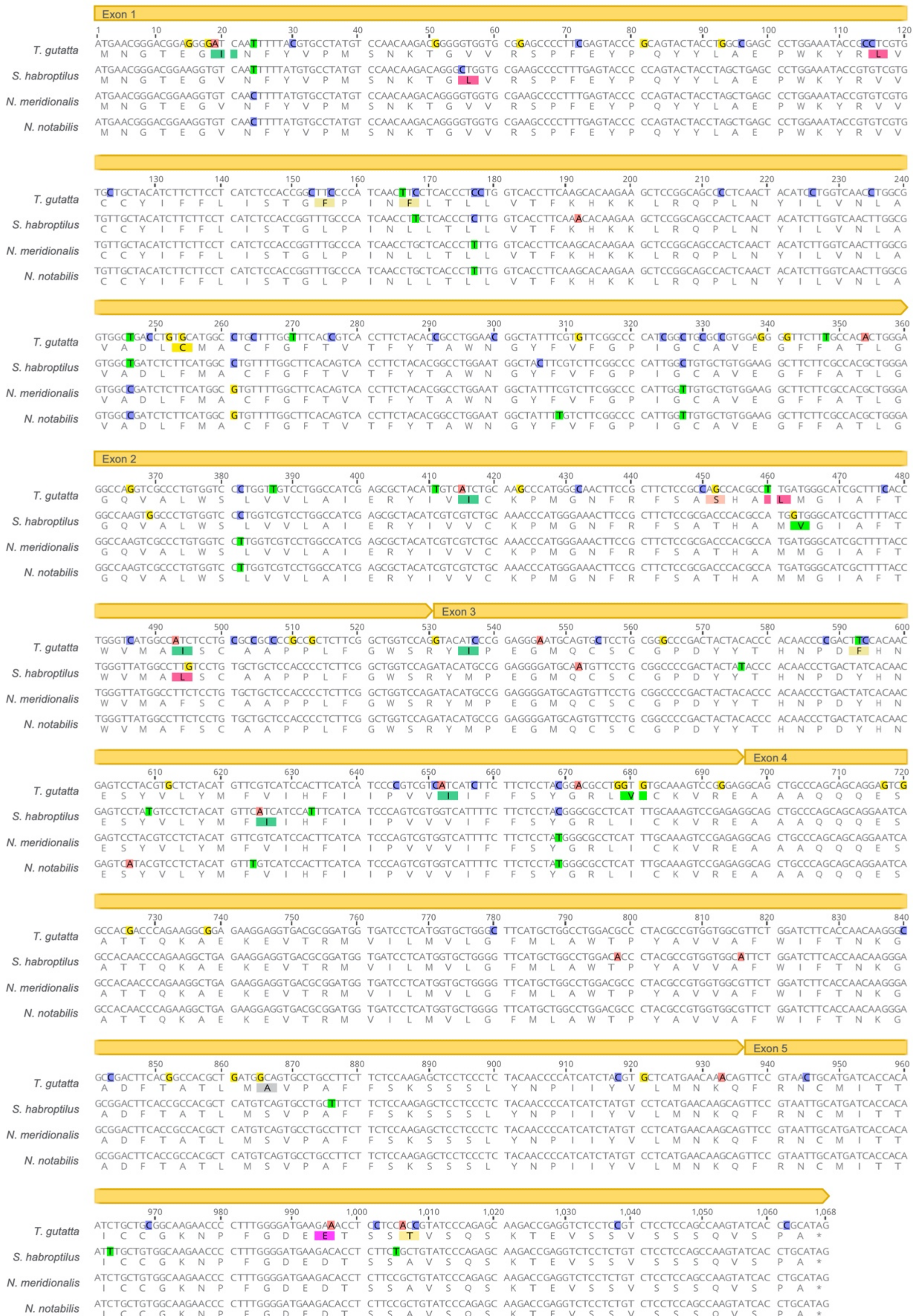


Figure S1. Partial nucleotide sequences alignment of the RH2 gene for the kea reference assembly and seven kea samples ascertained with Sanger sequencing (exon 3, intron 3 and partial exon 4). The yellow bar above the sequence indicates the start and stop position of the exons and intron. Coloured nucleotides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



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426 **Figure S2.** RH2 gene CDS nucleotide alignment (and amino acid translation) for zebra finch,

427 kākāpō, kākā, and kea (the later sequences have been corrected based on the results of this

428 study). The yellow bar above the sequence indicates the start and stop position of the exons.

429 Coloured nucleotides and amino acids highlight polymorphisms between the different species.