

1 **Title**

2 Olfactory receptor subgenome and expression in a highly olfactory procellariiform seabird

3

4 **Authors**

5 Simon Yung Wa Sin<sup>1,2,4\*</sup>, Alison Cloutier<sup>1,4</sup>, Gabrielle Nevitt<sup>3</sup>, and Scott V. Edwards<sup>1</sup>

6

7 **Author affiliation**

8 <sup>1</sup> Department of Organismic and Evolutionary Biology, Museum of Comparative Zoology,  
9 Harvard University, 26 Oxford Street, Cambridge, MA 02138, USA

10 <sup>2</sup> School of Biological Sciences, The University of Hong Kong, Pok Fu Lam Road, Hong Kong  
11 SAR

12 <sup>3</sup> Department of Neurobiology, Physiology and Behavior and the Graduate Group in Ecology,  
13 University of California, Davis, CA 95616, USA

14 <sup>4</sup> These authors contributed equally.

15

16 **\*Author for correspondence:**

17 Simon Yung Wa Sin, School of Biological Sciences, The University of Hong Kong, Pok Fu  
18 Lam Road, Hong Kong SAR

19 Telephone number: (852)22990825

20 Email: [sinyw@hku.hk](mailto:sinyw@hku.hk)

21

22 **Running title**

23 OR genes in a storm petrel

## 24 **Abstract**

25           Procellariiform seabirds are known for their well-developed olfactory capabilities,  
26 reflected by their large olfactory bulb to brain ratio and olfactory-mediated behaviors. Many  
27 species in this clade use olfactory cues for foraging and navigation, and some species can  
28 recognize individual-specific odors. Their genomes and transcriptomes may yield important  
29 clues about how the olfactory receptor (OR) subgenome was shaped by natural and sexual  
30 selection. In this study, we assembled a high-quality Leach's storm petrel (*Oceanodroma*  
31 *leucorhoa*) genome to facilitate characterization of the OR repertoire. We also surveyed  
32 expressed OR genes through transcriptome analysis of the olfactory epithelium - to our  
33 knowledge, the first avian study to interrogate OR diversity in this way. We detected a large  
34 number (~61) of intact OR genes, and identified OR genes under positive selection. In  
35 addition, we estimated that this species has the lowest proportion (~60%) of pseudogenes  
36 compared to other waterbirds studied thus far. We show that the traditional annotation-based  
37 genome mining method underestimates OR gene number (214) as compared to copy number  
38 analysis using depth-of-coverage analysis, which estimated a total of 492 OR genes. By  
39 examining OR expression pattern in this species, we identified highly expressed OR genes,  
40 and OR genes that were differentially expressed between age groups, providing valuable  
41 insight into the development of olfactory capabilities in this and other avian species. Our  
42 genomic evidence is consistent with the Leach's storm petrel's well-developed olfactory  
43 sense, a key sensory foundation for its pelagic lifestyle and behavioral ecology.

44

## 45 **Keywords**

46 Leach's storm petrel, *Oceanodroma leucorhoa*, sensory ecology, olfaction, olfactory receptor  
47 gene repertoire, transcriptome

## 48 **Introduction**

49           Animals have evolved different senses to survive and flourish in changing  
50 environments. Of the several animal senses, olfaction is the physiological function detecting  
51 highly diverse and abundant chemicals originating from the surrounding environment and  
52 other organisms. Olfaction is important for animals to recognize food, mates, relatives,  
53 offspring, predators, diseases, territories, and many other important functions (Wyatt 2003).  
54 It is therefore crucial for their survival and reproduction.

55           In vertebrates, the ability to detect and differentiate tens of thousands of odorants is  
56 largely mediated by olfactory receptors (ORs) expressed in the olfactory epithelium of the  
57 nasal cavity (Buck and Axel 1991). Olfactory receptors are transmembrane G protein-  
58 coupled receptors (GPCRs) with seven  $\alpha$ -helical transmembrane domains bound to a G-  
59 protein. The binding of extracellular ligands to ligand-binding sites of ORs triggers  
60 conformational changes that lead to intracellular signaling cascades, resulting in transmission  
61 to the olfactory bulb in the brain (Fredriksson, et al. 2003), which ultimately leads to  
62 olfactory perception. It has been proposed that different types of ligands are recognized by  
63 different combinations of ORs to enable an individual to perceive thousands of chemicals as  
64 distinct odors (Malnic, et al. 1999). The large number of ORs in vertebrates are classified into  
65 two groups. Class I ORs are hypothesized to bind water-borne hydrophilic ligands, and class  
66 II ORs appear to bind airborne hydrophobic ligands (Saito, et al. 2009).

67           Olfactory receptors are encoded by OR genes, which, at approximately 1,000 bp in  
68 size, are without introns and relatively short. OR genes are the largest multigene family in  
69 vertebrates (Nei, et al. 2008). Moreover, frequent gains and losses through duplication and  
70 pseudogenization, have resulted in dramatic differences in OR repertoire and gene number  
71 between species (Nei, et al. 2008; Nei and Rooney 2005; Niimura 2012). New OR families  
72 likely originate through gene duplication and positive selection leading to

73 neofunctionalization and species-specific adaptations, whereas loss of function of some gene  
74 duplicates typically results in a large number of OR pseudogenes (Innan 2009; Lynch and  
75 Force 2000). The number of intact OR genes ranges from 40 in pufferfish (Niimura and Nei  
76 2005) to ~2000 in the African elephant (Niimura, et al. 2014). The overall size and diversity  
77 of the OR repertoire across species is believed to be influenced by ecological adaptation and  
78 reliance on olfaction (Gilad, et al. 2004; Hayden, et al. 2010). Highly olfactory mammals  
79 such as elephants have many intact OR genes compared to primate species, such as  
80 macaques, which rely more on vision than olfaction whose genomes have a smaller number  
81 of intact OR genes and a larger proportion of pseudogenes (Matsui, et al. 2010; Niimura, et  
82 al. 2014).

83         Among vertebrates, birds are well-known for their excellent sense of vision whereas  
84 olfaction has been largely ignored by ornithologists. However, emerging evidence shows that  
85 many birds have well-developed olfactory abilities that likely rival many mammals, including  
86 humans (Bang 1966; Bonadonna and Nevitt 2004; Corfield, et al. 2015; Nevitt, et al. 2008;  
87 Roper 1999; Zelano and Edwards 2002). The OR repertoires of birds are small relative to  
88 many other vertebrates, and gains and losses and pseudogenization seems to play an  
89 important role in their evolution (Khan, et al. 2015; Organ, et al. 2010). Ecological factors  
90 and life-history adaptations appear to have shaped the olfactory abilities and repertoire  
91 variation among birds of prey, water birds, land birds, and vocal learners (Corfield, et al.  
92 2015; Khan, et al. 2015). Although there was an expansion in OR family 14 (the  $\gamma$ -c clade) in  
93 birds and the majority of avian OR genes belong to this family, some bird species and  
94 lineages exhibit alternative patterns of OR gene family expansions or reductions (Khan, et al.  
95 2015). For example, the estimated number of OR genes is larger in the nocturnal brown kiwi  
96 (*Apteryx australis*) and flightless parrot, kakapo (*Strigops habroptilus*), than in their diurnal  
97 relatives (Steiger, et al. 2009a). In contrast, penguins, like many aquatic mammals (Hayden,

98 et al. 2010), possess a high percentage of OR pseudogenes (Lu, et al. 2016), which appear to  
99 have been pseudogenized during the transition from a terrestrial to a marine habitat,  
100 suggesting that olfactory perception or use changed as well.

101 Olfactory ability is reflected by the olfactory bulb to brain ratio, which correlates  
102 positively with the estimated total number of OR genes in birds (Khan, et al. 2015; Steiger, et  
103 al. 2008). Among extant birds, the Procellariiformes, also called tube-nosed seabirds, which  
104 includes the storm-petrels, albatrosses, diving petrels, and shearwaters, have the largest  
105 olfactory bulb to brain ratio (Corfield, et al. 2015). These seabirds are known for their  
106 excellent olfactory ability. Many seabird species use olfactory cues to locate areas for  
107 foraging (Nevitt 1999a; Nevitt 2000; Nevitt 1999b; Nevitt, et al. 2004; Nevitt, et al. 1995),  
108 and several burrow-nesting species use odor to locate their burrow when returning to the  
109 colony after offshore foraging trips (Bonadonna and Bretagnolle 2002; Bonadonna, et al.  
110 2004). Additionally, some species can recognize individual-specific odors (Bonadonna and  
111 Nevitt 2004). Olfaction therefore plays a crucial role in survival and communication in this  
112 group of seabirds. Given the importance of olfaction and the large olfactory bulb in these  
113 birds, they are good candidates for studying the evolution of avian OR genes.

114 Leach's storm-petrels *Oceanodroma leucorhoa* (Vieillot, 1818), a procellariiform  
115 seabird, rely heavily on their well-developed sense of smell for foraging, homing, and mate  
116 recognition. They can smell dimethyl sulfide (DMS) and use it as a foraging cue (Nevitt and  
117 Haberman 2003). Olfaction also plays a fundamental role in social communication and  
118 individual recognition in this species (O'Dwyer, et al. 2008). Their musky smelling plumage  
119 is imbued with volatile chemicals that may give them individual olfactory signatures. They  
120 are burrow-nesting and in general adults are faithful to their burrow and mate throughout  
121 their lifetime (Morse and Buchheister 1977). In each breeding season, a breeding pair raise a  
122 single chick, which remains in the egg for 45 days and in the burrow until it fledges 60 days

123 old to forage at sea (Warham 1990) – a remarkable life history for a bird weighing only ~47  
124 g. Each burrow has its own unique olfactory signature, and chicks can recognize and prefer  
125 familiar odors of their natal burrow (O'Dwyer, et al. 2008). It is suggested that a memory for  
126 familial odors may play a role later in life in the context of kin recognition and mate choice  
127 (O'Dwyer, et al. 2008). Although links to individual odor profiles have not yet been  
128 established, MHC-based mate choice by males has been recently demonstrated in this species  
129 (Hoover, et al. 2018), making it an ideal candidate for the study of OR repertoire and  
130 evolution.

131         Here we sequenced and assembled a high-quality genome of the Leach's storm-petrel  
132 and characterized its OR gene family repertoire, allowing us to measure expansion and  
133 turnover in OR gene families in this procellariiform seabird and relatives. In most studies  
134 attempting to identify OR genes using genome-mining techniques such as BLAST, the sizes  
135 of OR repertoires are likely underestimated because of the collapse of similar OR sequences  
136 during assembly (Khan, et al. 2015; Sudmant, et al. 2010). We therefore also estimated the  
137 copy number (Malmstrøm, et al. 2016; Sudmant, et al. 2010) of the identified OR sequences  
138 in an effort to obtain a more accurate estimate of OR gene number. Whole-genome  
139 sequencing is the best approach to study the evolution of this large multigene family (Dehara,  
140 et al. 2012; Khan, et al. 2015; Matsui, et al. 2010; Niimura, et al. 2014; Vandewege, et al.  
141 2016). However, at present, the northern fulmar (*Fulmarus glacialis*) is the only  
142 procellariiform species with a sequenced genome, which lacks high contiguity (contig N50 =  
143 26k) and completeness (>10% universal single-copy orthologs missing) compared to other  
144 genomes analyzed thus far (Khan, et al. 2015). The northern fulmar genome is therefore not  
145 ideal for the identification of OR genes and estimation of OR gene copy numbers. In  
146 addition, the life-history and foraging strategies of northern fulmars are very different from  
147 Leach's storm petrels. Northern fulmars are surface-nesting, which is a derived trait

148 compared to most other burrow-nesting procellariiform species (van-Buskirk and Nevitt  
149 2008). The nesting behavior has also evolved in conjunction with responsiveness to olfactory  
150 cues and foraging style (van-Buskirk and Nevitt 2008), and olfaction is likely to be the  
151 dominant sense in burrow-nesting species such as the Leach's storm petrel.

152 In addition to interrogating the Leach's storm-petrel genome, we investigated the  
153 expression of OR genes in the olfactory epithelium. Procellariiform seabirds have well-  
154 developed olfactory concha (Bang 1966) where the interaction of ORs with ligands and  
155 detection of odors takes place. However, to our knowledge, there is currently no study of OR  
156 transcriptomes in birds, including chicken. Most OR genes have been identified through  
157 comparative genomic techniques using homology searches to annotate protein coding  
158 sequences, but there is typically no experimental data to support whether identified OR genes  
159 are actually expressed in the olfactory epithelium in birds. ORs are also expressed in non-  
160 olfactory tissues (Fukuda, et al. 2004; Pluznick, et al. 2009) and in sperm (Spehr, et al. 2003).  
161 Hence it is possible that some OR genes are not expressed in olfactory epithelium and play  
162 no role in the sense of smell. In addition, the difference in expression level of different OR  
163 genes and families is unknown even for those genes that are expressed in olfactory tissues.  
164 The relationship between expression pattern and function in life-history is also important to  
165 understand olfactory-mediated behaviors. If there were sexual dimorphism or developmental  
166 differences in olfactory-mediated behaviors, OR gene expression may facilitate these  
167 differences. To study OR expression, we used transcriptome sequencing (RNA-seq) to  
168 compare OR gene expression between male and female birds, and between adults and chicks,  
169 allowing us to identify highly expressed OR genes, and OR genes differentially expressed  
170 between age classes.

171

172 **Materials and Methods**

173 ***Sample collection***

174 We captured Leach's storm-petrels (n = 10) at Bon Portage Island, Nova Scotia,  
175 Canada (43°26' N, 65°45' W), where approximately 50,000 pairs breed annually (Oxley  
176 1999). The age class (chick or adult) and burrow number of each individual were recorded  
177 (Hoover, et al. 2018). Approximately 75 µl of blood was taken from one male via brachial  
178 venipuncture and stored in a microcentrifuge tube containing Queen's lysis buffer (Seutin, et  
179 al. 1991) and were then stored unfrozen at 4°C until DNA extraction for whole-genome  
180 sequencing. The anterior olfactory concha and right brain were collected from three adult  
181 females, three adult males, and three chicks during August, 2015, and were stored in  
182 RNAlater at 4°C for a few days until RNA extraction. All sampling was conducted in  
183 adherence to guidelines defined by the University of California, Davis Institutional Animal  
184 Care and Use Committee Protocol #19288, and Canadian Wildlife Service (permit #SC2792).

185 ***DNA extraction and whole-genome sequencing***

186 We isolated genomic DNA using the DNeasy Blood and Tissue Kit (Qiagen, Hilden,  
187 Germany) and determined sex of the individual for whole-genome sequencing using  
188 published PCR primers (2550F & 2718R; Fridolfsson and Ellegren 1999). We measured  
189 DNA concentrations using a Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, USA) and  
190 performed whole-genome libraries preparation and sequencing following Grayson et al.  
191 (2017) on an adult male. In brief, a DNA library of 220 bp insert size was prepared using the  
192 PrepX ILM 32i DNA Library Kit (Takara), and mate-pair libraries of 3 kb and 6 kb insert  
193 sizes were prepared using the Nextera Mate Pair Sample Preparation Kit (cat. No. FC-132-  
194 1001, Illumina). We then assessed library quality using the HS DNA Kit (Agilent) and  
195 quantified the libraries with qPCR prior to sequencing (KAPA library quantification kit). We  
196 sequenced the libraries on an Illumina HiSeq instrument (High Output 250 kit, PE 125 bp  
197 reads) at the Bauer Core facility at Harvard University. We assessed the quality of the

198 sequencing data using FastQC, removed adapters using Trimmomatic (Bolger, et al. 2014),  
199 and assembled the genome using AllPaths-LG (Gnerre, et al. 2011). The completeness of the  
200 assembled genome was measured with BUSCO v2.0 (Simão, et al. 2015) and the aves\_odb9  
201 dataset to search for 4915 universal single-copy orthologs in birds.

### 202 ***RNA extraction and transcriptome sequencing***

203 RNA was extracted from each sampled tissue using RNeasy Plus Mini kit (Qiagen).  
204 The quality of the total RNA was assessed using the RNA Nano Kit (Agilent). Poly-A  
205 selection was conducted on the total RNA using the PrepX PolyA mRNA Isolation Kit  
206 (Takara). The mRNA was assessed using the RNA Pico kit (Agilent) and used to make  
207 transcriptome libraries using the PrepX RNA-Seq for Illumina Library Kit (Takara). The HS  
208 DNA Kit (Agilent) was used to assess library quality. The libraries were quantified by  
209 performing qPCR (KAPA library quantification kit) and then sequenced on a NextSeq  
210 instrument (High Output 150 kit, PE 75 bp reads). Each of a total of 29 libraries (Table S1)  
211 was sequenced to a depth of approximately 30M reads. The individuals for RNA-seq were  
212 not the same as the individual for whole-genome sequencing (Table S1).

### 213 ***Genome annotation***

214 We annotated the Leach's storm-petrel genome using MAKER v2.31.8 (Holt and  
215 Yandell 2011). We combined *ab initio* gene prediction with protein-based evidence from 16  
216 other vertebrates (10 birds, 3 reptiles, 2 mammals, and 1 fish species), as well as the  
217 transcriptome assembly and TopHat junctions from the Leach's storm-petrel (Table S1). We  
218 assembled the storm-petrel transcriptome from 10 tissues of a single individual (Table S1)  
219 using TRINITY 2.1.1 (Grabherr, et al. 2011) and inferred splice junctions using TopHat  
220 2.0.13 (Kim et al. 2013). We functionally annotated the genome to identify putative gene  
221 function and protein domains using NCBI BLAST+ and the UniProt/Swiss-Prot set of

222 proteins. We used BLASTP on the list of proteins identified by MAKER with an evaluate of  
223 1e-6.

## 224 ***Data analysis***

### 225 OR gene identification/annotation

226 We identified the OR genes in the Leach's storm-petrel genome assembly with  
227 TBLASTN searches using published intact OR amino acid sequences from Vanderwege et al.  
228 (2016), Niimura et al. (2009) and the HORDE database (The Human Olfactory Data  
229 Explorer). The queries include intact OR genes from 12 species of birds, reptiles, mammals,  
230 amphibians, and fish (Table S2). We first identified all high-scoring segment pairs (HSPs)  
231 with a minimum length of 150 bp and an e-value < 1e-10. We then used BEDTools intersect  
232 (Quinlan and Hall 2010) and custom Perl scripts to tile overlapping HSPs and remove  
233 redundant BLAST results to produce a set of candidate OR regions in the storm-petrel.

234 Candidate OR regions were manually reviewed to omit spurious (non-OR) hits and to  
235 determine if each region represents an intact OR gene, a pseudogene, a truncated OR  
236 sequence, or an OR gene fragment. The region spanning +/- 700 bp to each side of the  
237 predicted OR location was used in an online blastx search against the NCBI non-redundant  
238 database delimited by organism 'Aves'. Candidate OR genes were omitted if they had top  
239 BLAST hits to non-OR sequences (e.g. other non-OR GPCRs), and coordinates for retained  
240 genes were refined based on BLAST hits to other avian ORs.

241 OR genes were classified as 'intact' if they contained start and stop codons, with no  
242 internal stops or frameshifts, and as 'pseudogenes' if they covered the full coding region but  
243 contained internal stops or frameshifts, or had large (> 5 amino acids) insertions or deletions  
244 within transmembrane regions. Candidate ORs that spanned incomplete coding sequences  
245 were classified as 'truncated' if they abutted a scaffold edge or a gap between contigs, or as  
246 an OR gene 'fragment' if they had an apparently naturally incomplete coding region that was

247 not at a scaffold or contig edge. 'Truncated' or 'fragmented OR genes' could also be  
248 classified as 'pseudogenes' if they contained internal stops or frameshifts; OR genes could  
249 also be classified as both 'truncated' and 'fragmented' (e.g. truncated at one end and  
250 fragmented at other).

251 We performed a second TBLASTN search using the intact storm-petrel OR genes as  
252 queries to search back against the petrel genome assembly to identify any additional  
253 candidate regions that may have been missed in the first TBLASTN search. Candidate  
254 regions were compared to the OR genes identified in the first round of blast searching with  
255 the BEDTools subtract option, requiring 10% overlap. We then used NCBI's conserved  
256 domain search to annotate transmembrane regions TM1-TM7.

#### 257 Phylogenetic analysis and OR gene family assignment

258 We used phylogenetic analysis of OR amino acid sequences to compare intact storm-  
259 petrel OR genes to other avian and reptilian OR genes. The result was used primarily to  
260 assign Leach's storm-petrel genes to an OR subfamily. We included intact OR sequences  
261 from the American alligator, green anole, chicken, and zebra finch from Vanderwege et al.  
262 (2016), and waterbirds, including members of Sphenisciformes, Pelecaniformes, Suliformes,  
263 Gaviiformes, Phoenicopteriformes, Podicipediformes, and Anseriformes, with assembled  
264 genomes and annotated gene models on NCBI (Jarvis, et al. 2014) (Table S3). Pseudogenes,  
265 genes encoded by multiple exons, truncated genes, and partial coding regions (< 275 AA)  
266 were omitted. We used five non-OR rhodopsin family GPCRs from chicken as outgroups  
267 (Niimura 2009). They are alpha-1A adrenergic receptor (ADRA1A), 5' hydroxytryptamine  
268 receptor 1B (HTR1B), somatostatin receptor type 4 (SSTR4), dopamine receptor D1  
269 (DRD1), and histamine receptor H2 (HRH2).

270 We aligned the sequences with the 'einsi' option in MAFFT v. 7.407. We manually  
271 reviewed the alignment and removed sequences with large indels (> 10 consecutive amino

272 acids). We also removed duplicates and any sequences with > 5% uncalled residues (Xs), or  
273 > 10 Xs in total, unless they were Leach's storm-petrel OR genes or outgroup sequences. We  
274 aligned the retained sequences again with the MAFFT *einsi* option as described above,  
275 following which the alignment edges were trimmed to retain only the region spanning  
276 transmembrane regions TM1-TM7 for phylogenetic analysis.

277 We used ProTest3 v.3.4.2 (Darriba, et al. 2011) to determine the best-fitting model of  
278 amino acid substitution, which was JTT + G + F. The best maximum-likelihood topology was  
279 inferred with RAxML v. 8.2.10 (Stamatakis 2014) from 100 searches, each starting from a  
280 different random starting tree. Five hundred bootstrap replicates were computed with  
281 RAxML, and the bootstraps were plotted on the bestML tree. The bestML + bootstraps tree  
282 was then rooted on the chicken non-OR outgroups with ETE3 (Huerta-Cepas, et al. 2016).  
283 The final tree was visualized in MEGA X (Tamura, et al. 2011). Leach's storm-petrel genes  
284 were then assigned to an OR family based on phylogenetic relationships.

#### 285 OR gene copy number analysis

286 We calculated the genomic depth-of-coverage (DoC) for each olfactory receptor gene  
287 identified in the Leach's storm-petrel genome assembly. We then compared each DoC to the  
288 genome-wide DoC to determine if any predicted OR genes represented collapsed gene copies  
289 in the genome assembly (Malmström, et al. 2016; Sudmant, et al. 2010). We could then  
290 estimate the total expected number of petrel ORs. We first repeatmasked the reference  
291 genome assembly with query species 'vertebrata metazoa' using RepeatMasker v. 4.0.5 (Smit,  
292 et al. 2015) with RepeatMasker Library 'Complete Database 20160829'. The reads of the  
293 220bp fragment libraries were trimmed with Trimmomatic v. 0.32 (Bolger, et al. 2014) and  
294 mapped to the storm-petrel genome assembly using BWA v. 0.7.15 (Li and Durbin 2010)  
295 with default parameters. SAMtools v. 1.5 (Li, et al. 2009) was used to post-process mapped  
296 reads and merge output BWA SAM files. Reads that were unmapped or below the minimum

297 mapping quality of ‘30’ were omitted. Duplicates were marked and removed with Picard v.  
298 2.18.9 (<https://broadinstitute.github.io/picard/>). Per-base depth of coverage was then output  
299 with the BEDTools v. 2.26.0 genomecov option.

300 To incorporate the difference in DoC due to variable GC content for the estimation of  
301 OR gene copy number, we used the repeatmasked reference genome to calculate DoC for  
302 non-repetitive regions only. We calculated DoC within bins of 1000 bp (approximately the  
303 size of an intact OR gene) with at least 98% base (non-N) occupancy. For each bin, we  
304 calculated the %GC and the average DoC. Then we calculated the mean DoC within each  
305 bin, and placed bins in categories of 5% GC (e.g. 0-5%, 5-10%, 10-15%, etc.). We took the  
306 ratio of each Leach’s storm-petrel OR gene DoC and compared it to the estimated DoC for  
307 the bins with similar GC content. This DoC analysis could not be done for other waterbirds  
308 because genome coordinates for intact, pseudo- and truncated OR genes are needed, but they  
309 are not provided in Khan et al. (2015).

#### 310 OR gene expression analysis

311 We assessed the quality of the RNA-seq data using FastQC (Andrews 2010). We  
312 performed error correction using Rcorrector and removed unfixable reads using a custom  
313 python script  
314 (<https://github.com/harvardinformatics/TranscriptomeAssemblyTools/blob/master/FilterUnco>  
315 [rrectabledPEfastq.py](https://github.com/harvardinformatics/TranscriptomeAssemblyTools/blob/master/FilterUnco)). We next removed adapters and low quality reads (-q 5) using  
316 TrimGalore! v0.4 (Krueger 2016). We removed reads of rRNAs by mapping to the Silva  
317 rRNA database using Bowtie2 2.2.4 (Langmead and Salzberg 2012) with the --very-  
318 sensitive-local option, and retained reads that did not map to the rRNA database.

319 We used RSEM (v1.2.29) (Li and Dewey 2011) to quantify levels of gene expression.  
320 We first built an RSEM index for the annotated Leach’s storm-petrel genome, then used  
321 RSEM to implement Bowtie2 (v2.2.6) for the mapping of RNA-seq reads to the genome,

322 using default parameters for mapping and expression quantification. Expected read counts  
323 per million at the gene level from RSEM were used to represent the normalized expression.  
324 We used the normalized counts rounded from RSEM outputs as inputs for differential  
325 expression analysis. We then used limma voom (Law, et al. 2014) to identify differentially  
326 expressed genes between adults and chicks, and between male and female adults, using a 5%  
327 FDR cutoff.

### 328 Gene ontology (GO) analysis

329 We used GOrilla to perform GO analysis (Eden, et al. 2009), using the single ranked  
330 list of genes mode. Reported enrichment  $p$  values were FDR-adjusted using the Benjamini–  
331 Hochberg method (Benjamini and Hochberg 1995).

### 332 Analysis of positive selection on OR family 14

333 We detected sites that were under selection by investigating the ratio of the rate of  
334 synonymous substitutions to the rate of non-synonymous substitutions ( $\omega = dN/dS$ ), which  
335 may indicate positive selection ( $\omega > 1$ ), neutral ( $\omega = 1$ ), or negative selection ( $\omega < 1$ ). We  
336 used the HyPhy package (Pond and Muse 2005) implemented in the Datamonkey webserver  
337 ([datamonkey.org](http://datamonkey.org)) to infer potential recombination breakpoints and estimate  $\omega$ . Since  
338 recombination and gene conversion can mislead estimation of selection, we used Genetic  
339 Algorithm for Recombination Detection (GARD) (Pond, et al. 2006) to generate multiple  
340 phylogenies based on putative non-recombinant fragments. We then used Single-Likelihood  
341 Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), Mixed Effects Model of  
342 Evolution (MEME), and Fast Unconstrained Bayesian AppRoximation (FUBAR) methods  
343 implemented in HyPhy, plus an integrated approach that incorporates all sites detected by  
344 each method, to infer signals of positive selection. Here, sites detected by two or more  
345 methods are considered under selection. All methods were used with default settings. We

346 used WebLogo ([weblogo.threeplusone.com](http://weblogo.threeplusone.com)) to visualize the amino acid sequence variation of  
347 the transmembrane (TM), intracellular (IC) and extracellular (EC) domains.

348

## 349 **Results**

### 350 Assembly of Leach's storm-petrel genome

351 We generated 439,914,448 reads from the 220 bp library, 313,504,024 reads from the  
352 3 kb library, and 269,594,574 reads from the 6 kb library. The genome size estimated by  
353 AllPaths-LG from k-mers is 1.24 Gb (Table 1). The contig N50 is 165.4 kb and the scaffold  
354 N50 is 8.7 Mb (Table 1). BUSCO (Simão, et al. 2015) shows a high completeness of the  
355 genome, with 98.0% of single-copy orthologs for birds identified and 94.7% represented by  
356 complete coding sequences in the genome (Table 1). The MAKER run identified a total of  
357 15510 gene models. The genome-wide GC content is 42.1%.

358

### 359 OR genes in Leach's storm petrel

360 We identified 221 candidate OR regions from the initial round of TBLASTN (Table  
361 2). Eight of these regions were not ORs. The second TBLASTN search using all identified  
362 intact OR genes as queries identified one additional pseudogene fragment region not found in  
363 the initial round of search, yielding 214 OR regions in total. Of these 214 OR regions, 61  
364 (28.5%) were intact OR genes, and the remainder included 106 pseudogenes (49.5%), 20  
365 truncated genes (9.3%), and/or 27 gene fragments (12.6%) (Table 2; Fig. 1).

366 To estimate the total number of OR genes, we incorporated the number of collapsed  
367 gene copies for the 214 identified OR genes. By calculating the ratio of each OR gene DoC to  
368 the estimated DoC for bins of similar GC content across the storm-petrel genome (Fig. S1),  
369 we estimated there are as many as 492 predicted OR genes in the Leach's storm-petrel  
370 genome (Table 2). As expected, genes in high GC bins (> 50% GC) had lower coverage than

371 genes in low GC bins (< 45%; Botero-Castro, et al. 2017). The average estimated copy  
372 number for intact OR genes was 2.7 and the total number of intact OR genes was 163  
373 (33.1%) (Table 2). The copy number of intact OR genes ranged from 1 to 45 (mean = 2.7, SD  
374 = 5.8) (Table S4). Of the 24 intact OR genes with multiple copies, 13 belonged to OR family  
375 14 ( $\gamma$ -c clade; Khan, et al. 2015), which included the intact gene with the highest copy  
376 number ratio of 45. The total number of estimated pseudogenes, truncated genes, and gene  
377 fragments was 224 (45.5%), 51 (10.4%), and 54 (11%), respectively (Table 2; Fig. 1).

378

### 379 OR gene family phylogeny

380 We performed phylogenetic analyses using all intact OR genes from the Leach's  
381 storm-petrel genomes and 13 waterbirds, plus ORs from American alligator, green anole,  
382 chicken, and zebra finch. We found that sequences largely cluster by OR gene family,  
383 although typically with low bootstrap support. Nevertheless, we were able to confidently  
384 assign 60 of 61 intact storm-petrel ORs to their OR gene family. The resulting phylogeny  
385 implied 10 OR gene families in the Leach's storm-petrel genome (Fig. 2), corresponding to  
386 numbers 2, 4, 5, 6, 8, 10, 13, 14, 51, and 52 in chicken.

387

### 388 Differential OR gene expression

389 We compared the patterns of OR gene expression in the olfactory concha, where OR  
390 genes are expected to be predominantly expressed, and in the brain, where we expect little  
391 OR gene expression (Fig. 3). Two OR genes were highly expressed in the olfactory  
392 epithelium: OR gene OR6-6 (OR family 6) and OR5-11 (OR family 5). Both OR genes had a  
393 copy number ratio of two. We found no differentially expressed OR genes in the olfactory  
394 epithelium between male and female adults (Fig. S2), but identified four OR genes  
395 differentially expressed between age classes: OR14-14, OR14-12, OR10-2, and OR14-9 (Fig.

396 3). The most differentially expressed OR gene, OR14-14, is also the OR gene with the  
397 highest copy number ratio at 45 (Table S4). OR14-12 and OR14-9 also had a relatively high  
398 copy number ratio at 5 and 9, respectively (Table S4). The two highly expressed ORs and  
399 four differentially expressed ORs are all class II ORs. In contrast to the expression in the  
400 olfactory epithelium, most OR genes were not expressed or exhibited minimal (~0)  
401 expression in the brain (Fig. 3C), and there were no differentially expressed OR genes in the  
402 brain sample. Gene ontology (GO) analyses of 6101 genes significantly differentially  
403 expressed (FDR < 0.01) in the olfactory epithelium between age classes revealed categories  
404 related to tissue growth and development, such as ossification and collagen fibril  
405 organization, as the most significantly enriched (Table S5). There were only 28 genes  
406 differentially expressed between adult males and females in the olfactory epithelium, with no  
407 GO categories enriched.

408

#### 409 OR genes under positive selection

410 We found evidence of two recombination breakpoints at nucleotide position 321 and  
411 450 of the alignment, located in the TM3 and TM4 domains, respectively (Fig. 4). Based on  
412 the inferred breakpoints, we used three data partitions to identify sites under selection in the  
413 intact genes of OR family 14. We identified signals of positive selection in OR family 14  
414 using multiple approaches. Although the overall  $\omega$  was 0.449 (SLAC), 0.436 (FEL), and  
415 0.449 (MEME), which suggest no evidence of positive selection across the genes as a whole,  
416 we detected signals of positive selection in individual codons. We identified codon positions  
417 4 and 107 (in TM3 domain) to be under positive selection using all methods (Table 3; Fig. 4).  
418 Codon positions 156 (in TM4), 200 (in TM5), and 250 (in TM6) were also under positive  
419 selection, identified by at least two methods (Table 3; Fig. 4).

420

## 421 **Discussion**

422           Our high-quality genome of a Leach's storm-petrel has higher contiguity than many  
423 bird genomes produced with short-read technology and allowed us to identify 61 intact OR  
424 genes and to estimate the proportion of intact and pseudogenized ORs. Because highly  
425 similar sequences from short-read libraries often lead to misassembled genes during whole-  
426 genome assembly (Alkan, et al. 2011), we examined the copy number ratio of OR sequences  
427 using depth-of-coverage and estimated a more than two-fold increase in OR gene number as  
428 compared to the annotation-only method. The OR gene number estimate incorporating the  
429 copy number ratio should be closer to the actual number of OR genes in this species  
430 (Malmstrøm, et al. 2016; Sudmant, et al. 2010). The actual number of OR genes is probably  
431 underestimated in most studies using genome blast-based mining and annotation only method  
432 to identify highly similar duplicated genes, a situation similar to the case of highly duplicated  
433 MHC genes (Malmstrøm, et al. 2016). Mapping of sequencing reads to estimate the copy  
434 number ratio is one way to better estimate the actual gene copy number (Malmstrøm, et al.  
435 2016). A limitation of this approach, however, is that the sequencing reads are usually shorter  
436 than the assembled OR sequences in the reference genome, and the highly similar nature of  
437 OR sequence also makes mapping assignment difficult or impossible, therefore the mapping  
438 depth-of-coverage for each OR sequence may deviate from the actual copy number ratio.  
439 Nonetheless, the total OR copy number estimate should be more accurate using this approach  
440 than genome mining alone. To provide a more accurate copy number estimation in the future,  
441 the emerging strategies using long-read sequencing technology that generates tens of  
442 kilobases read length can aid the study of multigene families such as OR genes (Miller, et al.  
443 2017).

444           When compared to other waterbirds (Khan, et al. 2015), the number of intact genes in  
445 Leach's storm-petrels is the highest if we consider the estimated copy number (Fig. 1). It is

446 also among the highest in intact OR number even when estimates of copy number are not  
447 considered, and is less than only one waterbird, the little egret (Fig. 1). The proportion of  
448 pseudogenes (pseudogene/(pseudogene+intact gene)) is the lowest among waterbirds, at  
449 approximately 60% in the Leach's storm-petrel compared to 69%-87% in other waterbirds  
450 (Fig. 1). Despite being the sister group to the Procellariiformes, the penguins  
451 (Sphenisciformes), represented here with Adelie and emperor penguins, are among the  
452 species with the lowest number of intact genes and the highest proportion of pseudogenes.  
453 This pattern may be due to their obligate mode of foraging underwater via diving behavior  
454 (Lu, et al. 2016). Another procellariiform seabird, the northern fulmar, has a similarly low  
455 number of intact genes and high proportion of pseudogenes as in penguins. One possibility is  
456 that the sequencing depth and genome assembly quality of the northern fulmar is much lower  
457 than that of the Leach's storm-petrel sequenced here, because the number of OR genes  
458 identified in the chicken and zebra finch, which have high-quality assembled genomes, was  
459 larger. However, the quality of the fulmar genome is comparable to many other waterbird  
460 genomes, and Khan et al. (2015) showed that there was no correlation between the number of  
461 OR genes identified and genome-wide sequencing depth. The high OR gene number in  
462 chicken (266 intact genes; 39.4% pseudogene) and zebra finch (190 intact genes; 61.7%  
463 pseudogene) may be due to species- or lineage-specific expansion in these groups (Fig. S3)  
464 (Khan, et al. 2015). Perhaps the different OR repertoires of the Leach's storm petrel and other  
465 waterbirds is a real biological signal that arose during the diversification of OR genes in  
466 different bird lineages.

467         The larger number of intact OR genes and smaller percentage of pseudogenized ORs  
468 in Leach's storm-petrels than most waterbirds suggests enhanced olfactory capabilities,  
469 consistent with the large olfactory bulb ratio in Procellariiformes (Corfield, et al. 2015; Khan,  
470 et al. 2015; Steiger, et al. 2008), and is supported by behavioral tests revealing a well-

471 developed sense of smell in this species (Nevitt and Haberman 2003; O'Dwyer, et al. 2008).  
472 Gene gains and losses through gene duplication and pseudogenization are the main processes  
473 in OR evolution among birds and other vertebrates (Khan, et al. 2015; Lu, et al. 2016;  
474 Niimura, et al. 2014; Steiger, et al. 2009b). The use of olfaction for behaviors such as  
475 foraging, homing, and mate recognition in the Leach's storm-petrel could be the selective  
476 force driving the evolution of OR gene number in this species. Being exclusively pelagic,  
477 procellariiforms are adapted to forage efficiently in order to survive in a vast area of open  
478 ocean where food sources can be patchy, unpredictable and transient. Explanation of OR  
479 gene number involving foraging habitat is not universal when we consider the low number of  
480 intact ORs in the northern fulmar, the only other Procellariiform with its genome sequenced  
481 and OR genes studied. There is a diversity of behaviors and ecologies among Procellariiform  
482 species. For example, Leach's storm petrels incubate their eggs and feed their chicks inside  
483 an underground burrow, whereas the northern fulmar nest on the ground (van-Buskirk and  
484 Nevitt 2008). Leach's storm petrel chicks spend almost the entire nestling period  
485 underground before they fledge, and parents enter and exit the breeding colony to feed their  
486 chicks nocturnally, when predation risk is lower. This difference in rearing environment  
487 could lead to differences in sensory functions (van-Buskirk and Nevitt 2008). A strong  
488 reliance on olfaction and good sense of smell may develop in Leach's storm-petrels being  
489 raised in darkness, whereas Procellariiform species exposed to more light may depend less on  
490 olfaction for homing and individual recognition (Mitkus, et al. 2016; Mitkus, et al. 2018).  
491 The Leach's storm-petrel indeed has six times lower visual spatial resolution than the  
492 northern fulmar (Mitkus, et al. 2016), which rely more on using vision than olfaction for  
493 foraging (van-Buskirk and Nevitt 2008). By investigating the OR subgenome in this study,  
494 our genomic and transcriptomic evidence confirms that the Leach's storm-petrel has superior  
495 olfactory capabilities among waterbirds and birds in general. Future studies should focus on

496 the relationship between OR repertoire and species-specific behavioral ecology in a wider  
497 and more densely sampled phylogenetic context to understand how natural and sexual  
498 selection shapes avian OR evolution.

499         Although the phylogenetic analysis did not reveal obvious species-specific expansion  
500 of a particular OR gene family in this species (but we cannot rule this out because we do not  
501 know if the highly duplicated gene copies would have orthologs in other species), several OR  
502 genes and domains experienced positive selection. We identified five amino acid sites under  
503 positive selection on OR family 14, the family that underwent rapid expansion in birds and  
504 showed signals of positive selection in eight other bird species (Khan, et al. 2015). Four of  
505 the five positively selected sites were located in transmembrane domains 3, 4, 5, and 6. These  
506 regions were also found to be highly variable in other species, and were suggested to  
507 participate in ligand binding (Niimura 2012; Quignon, et al. 2005). Specific genes belonging  
508 to OR family 14 had a high copy number when we examined the depth of coverage. This  
509 family belongs to class II ORs that bind airborne hydrophobic ligands and probably play a  
510 crucial role in the olfactory sense of this species, given the high number of copies in the  
511 genome.

512         OR genes experiencing substantial duplications, in particular OR14-14, suggest their  
513 high relevance to the ecology of Leach's storm-petrel. Identification of specific ligands for  
514 these ORs will help clarify the driving force for increasing gene copy number. For example,  
515 they may be important for foraging if OR14-14 or other OR 14-family genes bind dimethyl  
516 sulfide (DMS) or other ligands used in foraging (Nevitt, et al. 1995), or for communication  
517 and recognition if they bind odorants produced by other individuals. It is well known that  
518 individual olfactory sensory neurons (OSN) express a single OR allele out of hundreds of loci  
519 and alleles in the genome (Khamlichi and Feil 2018; Monahan and Lomvardas 2015). This  
520 monoallelic expression of OR genes determines the olfactory sensitivity of the neuron,

521 determining the ligands that will stimulate it. The single expressed OR also instructs axonal  
522 connections of the OSN to a specific glomerulus in the olfactory bulb. Expression of more  
523 than one OR allele may lead to disruption of olfactory network wiring and misinterpretation  
524 of the sense of smell (Magklara and Lomvardas 2013). The expression mechanism of a single  
525 OR per neuron is stochastic, initiated by random chromatin-mediated activation of a single  
526 OR expression and a feedback loop that stabilizes the initial OR and prevents additional OR  
527 allele expression (Chess 2012; Eckersley-Maslin and Spector 2014; Magklara and Lomvardas  
528 2013). Under this random monoallelic expression, an OR gene with more copies in the  
529 genome should have a larger representation in the OSN population than OR genes with a low  
530 copy number. Decoding and deorphanizing those highly duplicated ORs is a fascinating area  
531 for future research linking the olfactory environment, behavior and OR evolution.

532 To confirm that the identified intact OR genes are actually expressed in the olfactory  
533 epithelium we studied the transcriptome of the anterior olfactory concha. The intact OR  
534 genes identified transcriptomically were expressed in the olfactory epithelium, and different  
535 ORs were expressed at different levels. OR expression was almost absent in the brain sample,  
536 which likely included several subportions of the storm-petrel brain, including the olfactory  
537 bulb. The pattern of OR expression supports the role of identified OR genes in the detection  
538 of smell. To our knowledge, ours is the first study to investigate OR expression in the  
539 olfactory epithelium of birds using a transcriptomic approach. In other studies, once OR  
540 genes are identified by genome mining methods, there is often little confirmation to support  
541 the expression of OR genes in the olfactory epithelium. Interpreting OR gene evolution and  
542 understanding their relevance to sensory behavior may be hampered by the assumption that  
543 all annotated OR genes play a role in the sense of smell. By determining the expression of  
544 OR genes in different body tissues, we will be able to refine the functional interpretation of  
545 different OR genes, which may have roles outside of smell (Fukuda, et al. 2004; Pluznick, et

546 al. 2009; Spehr, et al. 2003). The differences in OR expression level among OR genes could  
547 be due to spatial patterning of OSN types in the olfactory epithelium (Coleman, et al. 2019).  
548 Now that we have identified the OR genes and transcripts in this study, future investigations  
549 can focus on the spatial and temporal patterns of OR gene expression, which is a research  
550 area currently lacking in birds, and has only been studied in a few non-avian model species  
551 such as mice (Coleman, et al. 2019; Hanchate, et al. 2015).

552 We found four OR genes that were differentially expressed in the olfactory epithelium  
553 between adults and chicks, belonging to families 14 and 10, both of which are class II ORs.  
554 All four genes were more highly expressed in chicks. Leach's storm-petrels can readily  
555 perform odor discrimination tasks as chicks soon after hatching (O'Dwyer, et al. 2008). A  
556 recent study by Mitkus et al. (2018) has shown that Leach's storm-petrel chicks are blind for  
557 the first 2 to 3 weeks post hatching suggesting a heightened reliance on olfaction. In our  
558 study, some of the most over-expressed genes we identified in chick compared to adult  
559 olfactory conchae are those that involved in ossification and soft tissue development (Table  
560 S5), such as the genes *SPARC*, *PHOSPHOI*, *Smpd3*, *COL1A1*, *COL1A2*, and *COL11A1*. The  
561 olfactory epithelium, as well as the sense of smell, of chicks sampled here were probably  
562 developing rapidly when sampled, perhaps resulting in higher expression levels of some OR  
563 genes in chicks than in adults. Alternatively, the lifespan of OSNs is affected by how  
564 frequently the ORs are used (Santoro and Dulac 2012). There is a mechanism to reduce the  
565 lifespan of OSNs that express infrequently used ORs (Santoro and Dulac 2012). This process  
566 can modulate the OSN population dynamics to adapt the olfactory system to a particular  
567 environment by changing the relative number of different types of OSNs, and the relative  
568 abundance of different OSNs changes with age and experience (Santoro and Dulac 2012;  
569 van-der-Linden, et al. 2018). Adult storm petrels that are foraging, navigating, homing, and  
570 recognizing mates, likely express a different repertoire of ORs than developing chicks, which

571 spend their entire early life inside their home burrows (but they are also interacting with the  
572 adults, feeding, walking around inside the burrow and they are very capable of discriminating  
573 different types of odors in choice tests). The difference in OR expression between chicks and  
574 adults might be caused by the difference in the usage frequency of different type of ORs,  
575 leading to variation in the lifespan and abundance of each type of OSN.

576 It has been proposed that MHC genes can affect body odor by changing the peptide  
577 community in the body (Brennan and Zufall 2006; Restrepo, et al. 2006). Alternatively, or in  
578 addition, individuals with different MHC genotypes may harbor different microbiome, which  
579 in turn produce different secondary metabolites and odor (Pearce, et al. 2017; Zomer, et al.  
580 2009). Highly diverse bacterial communities are often found in animal scent glands (Sin, et  
581 al. 2012; Theis, et al. 2013), and the uropygial gland of birds is one potential place that the  
582 secretion odor is affected by the microbiome it harbors (Rodríguez-Ruano, et al. 2015;  
583 Whittaker, et al. 2016). In Leach's storm-petrels, males appear to select their mates based on  
584 the MHC genotypes but females do not (Hoover, et al. 2018). Some insects using odors to  
585 select mates exhibit sexual dimorphism in the olfactory system (Brand, et al. 2018). The sex  
586 difference in MHC-based mate choice behavior in this species might be mediated through  
587 differentiated olfactory response to candidate mates with different body odors, which in turn  
588 could be due to intersexual differences in olfactory capabilities. An effect of MHC genes on  
589 body odor is yet to be shown in this species. Our study of gene expression in the olfactory  
590 epithelium revealed no intersexual differences in OR expression in adults. Thus, our study  
591 does not support the idea that intersexual differences in MHC-based mate choice behavior  
592 were due to different OR gene usages. However, this does not rule out that sexual  
593 dimorphism occurs in the olfactory center of the brain. Future studies of the relationship  
594 between MHC genotypes and body odor, and behavioral responses of birds to odors from

595 birds of different MHC genotypes, could help clarify whether mate choice in this species is  
596 mediated by olfaction.

597

## 598 **References**

599 Alkan C, Sajjadian S, Eichler EE 2011. Limitations of next-generation genome sequence  
600 assembly. *Nature Methods* 8: 61.

601 Andrews S 2010. FastQC: a quality control tool for high throughput sequence data. Available  
602 online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>.

603 Bang B 1966. The olfactory apparatus of tubenosed birds (Procellariiformes). *Cells Tissues  
604 Organs* 65: 391–415.

605 Benjamini Y, Hochberg Y 1995. Controlling the false discovery rate: a practical and  
606 powerful approach to multiple testing. *Journal of the Royal statistical society: series B  
607 (Methodological)* 57: 289–300.

608 Bolger A, Lohse M, Usadel B 2014. Trimmomatic: a flexible trimmer for Illumina sequence  
609 data. *Bioinformatics* 30: 2114–2120.

610 Bonadonna F, Bretagnolle V 2002. Smelling home: a good solution for burrow-finding in  
611 nocturnal petrels? *Journal of Experimental Biology* 205: 2519–2523.

612 Bonadonna F, Nevitt GA 2004. Partner-specific odor recognition in an Antarctic seabird.  
613 *Science* 306: 835.

614 Bonadonna F, Villafane M, Bajzak C, Jouventin P 2004. Recognition of burrow's olfactory  
615 signature in blue petrels, *Halobaena caerulea*: an efficient discrimination mechanism in  
616 the dark. *Animal Behaviour* 67: 893–898.

617 Botero-Castro F, Figuet E, Tilak M, Nabholz B, Galtier N 2017. Avian genomes revisited:  
618 hidden genes uncovered and the rates versus traits paradox in birds. *Molecular Biology  
619 and Evolution* 34: 3123–3131.

- 620 Brand P, Larcher V, Couto A, Sandoz J, Ramírez S 2018. Sexual dimorphism in visual and  
621 olfactory brain centers in the perfume-collecting orchid bee *Euglossa dilemma*  
622 (Hymenoptera, Apidae). *Journal of Comparative Neurology* 526: 2068–2077.
- 623 Brennan P, Zufall F 2006. Pheromonal communication in vertebrates. *Nature* 444: 308.
- 624 Buck L, Axel R 1991. A novel multigene family may encode odorant receptors: a molecular  
625 basis for odor recognition. *Cell* 65: 175–187.
- 626 Chess A 2012. Mechanisms and consequences of widespread random monoallelic expression.  
627 *Nature Reviews Genetics* 13: 421.
- 628 Coleman J, et al. 2019. Spatial determination of neuronal diversification in the olfactory  
629 epithelium. *Journal of Neuroscience* 39: 814–832.
- 630 Corfield J, et al. 2015. Diversity in olfactory bulb size in birds reflects allometry, ecology,  
631 and phylogeny. *Frontiers in Neuroanatomy* 9: 102.
- 632 Darriba D, Taboada G, Doallo R, Posada D 2011. ProtTest 3: fast selection of best-fit models  
633 of protein evolution. *Bioinformatics* 27: 1164–1165.
- 634 Dehara Y, et al. 2012. Characterization of squamate olfactory receptor genes and their  
635 transcripts by the high-throughput sequencing approach. *Genome Biology and Evolution*  
636 4: 602–616.
- 637 Eckersley-Maslin M, Spector D 2014. Random monoallelic expression: regulating gene  
638 expression one allele at a time. *Trends in Genetics* 30: 237–244.
- 639 Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z 2009. GOrilla: a tool for discovery and  
640 visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* 10: 48.
- 641 Fredriksson R, Lagerström M, Lundin L, Schiöth H 2003. The G-protein-coupled receptors in  
642 the human genome form five main families. Phylogenetic analysis, paralogon groups, and  
643 fingerprints. *Molecular Pharmacology* 63: 1256–1272.

- 644 Fridolfsson AK, Ellegren H 1999. A simple and universal method for molecular sexing of  
645 non-ratite birds. *Journal of Avian Biology* 30: 116–121.
- 646 Fukuda N, Yomogida K, Okabe M, Touhara K 2004. Functional characterization of a mouse  
647 testicular olfactory receptor and its role in chemosensing and in regulation of sperm  
648 motility. *Journal of Cell Science* 117: 5835–5845.
- 649 Gilad Y, Wiebe V, Przeworski M, Lancet D, Pääbo S 2004. Loss of olfactory receptor genes  
650 coincides with the acquisition of full trichromatic vision in primates. *PLoS Biology* 2:  
651 120–125.
- 652 Gnerre S, et al. 2011. High-quality draft assemblies of mammalian genomes from massively  
653 parallel sequence data. *Proceedings of the National Academy of Sciences* 108: 1513–  
654 1518.
- 655 Grabherr MG, et al. 2011. Full-length transcriptome assembly from RNA-Seq data without a  
656 reference genome. *Nature Biotechnology* 29: 644–652.
- 657 Grayson P, Sin S, Sackton T, Edwards S. 2017. *Comparative genomics as a foundation for  
658 evo-devo studies in birds*: Humana Press, New York, NY.
- 659 Hanchate N, et al. 2015. Single-cell transcriptomics reveals receptor transformations during  
660 olfactory neurogenesis. *Science* 350: 1251–1255.
- 661 Hayden S, et al. 2010. Ecological adaptation determines functional mammalian olfactory  
662 subgenomes. *Genome Research* 20: 1–9.
- 663 Holt C, Yandell M 2011. MAKER2: an annotation pipeline and genome-database  
664 management tool for second-generation genome projects. *BMC Bioinformatics* 12: 491.
- 665 Hoover B, et al. 2018. Ecology can inform genetics: Disassortative mating contributes to  
666 MHC polymorphism in Leach’s storm-petrels (*Oceanodroma leucorhoa*). *Molecular  
667 Ecology* 27: 3371–3385.

- 668 Huerta-Cepas J, Serra F, Bork P 2016. ETE 3: reconstruction, analysis, and visualization of  
669 phylogenomic data. *Molecular Biology and Evolution* 33: 1635–1638.
- 670 Innan H 2009. Population genetic models of duplicated genes. *Genetica* 137: 19.
- 671 Jarvis E, et al. 2014. Whole-genome analyses resolve early branches in the tree of life of  
672 modern birds. *Science* 346: 1320–1331.
- 673 Khamlichi A, Feil R 2018. Parallels between mammalian mechanisms of monoallelic gene  
674 expression. *Trends in Genetics* 34: 954–971.
- 675 Khan I, et al. 2015. Olfactory receptor subgenomes linked with broad ecological adaptations  
676 in Sauropsida. *Molecular Biology and Evolution* 32: 2832–2843.
- 677 Krueger F 2016. Trim Galore. Babraham Bioinformatics.
- 678 Langmead B, Salzberg SL 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods*  
679 9: 357–359.
- 680 Law C, Chen Y, Shi W, Smyth G 2014. voom: Precision weights unlock linear model  
681 analysis tools for RNA-seq read counts. *Genome Biology* 15: R29.
- 682 Li B, Dewey C 2011. RSEM: accurate transcript quantification from RNA-Seq data with or  
683 without a reference genome. *BMC Bioinformatics* 12: 323.
- 684 Li H, Durbin R 2010. Fast and accurate long-read alignment with Burrows–Wheeler  
685 transform. *Bioinformatics* 26: 589–595.
- 686 Li H, et al. 2009. The sequence alignment/map format and SAMtools. *Bioinformatics* 25:  
687 2078–2079.
- 688 Lu Q, Wang K, Lei F, Yu D, Zhao H 2016. Penguins reduced olfactory receptor genes  
689 common to other waterbirds. *Scientific Reports* 6: 31671.
- 690 Lynch M, Force A 2000. The probability of duplicate gene preservation by  
691 subfunctionalization. *Genetics* 154: 459–473.

- 692 Magklara A, Lomvardas S 2013. Stochastic gene expression in mammals: lessons from  
693 olfaction. *Trends in Cell Biology* 23: 449–456.
- 694 Malmstrøm M, et al. 2016. Evolution of the immune system influences speciation rates in  
695 teleost fishes. *Nature Genetics* 48: 1204.
- 696 Malnic B, Hirono J, Sato T, Buck L 1999. Combinatorial receptor codes for odors. *Cell* 96:  
697 713–723.
- 698 Matsui A, Go Y, Niimura Y 2010. Degeneration of olfactory receptor gene repertoires in  
699 primates: no direct link to full trichromatic vision. *Molecular Biology and Evolution* 27:  
700 1192–1200.
- 701 Miller J, et al. 2017. Hybrid assembly with long and short reads improves discovery of gene  
702 family expansions. *BMC Genomics* 18: 541.
- 703 Mitkus M, Nevitt G, Danielsen J, Kelber A 2016. Vision on the high seas: spatial resolution  
704 and optical sensitivity in two procellariiform seabirds with different foraging strategies.  
705 *Journal of Experimental Biology* 219: 3329–3338.
- 706 Mitkus M, Nevitt G, Kelber A 2018. Development of the Visual System in a Burrow-Nesting  
707 Seabird: Leach's Storm Petrel. *Brain, Behavior and Evolution* 91: 4–16.
- 708 Monahan K, Lomvardas S 2015. Monoallelic expression of olfactory receptors. *Annual*  
709 *Review of Cell and Developmental Biology* 31: 721–740.
- 710 Morse DH, Buchheister CW 1977. Age and survival of breeding Leach's storm-petrels in  
711 Maine. *Bird-Banding* 48: 341–349.
- 712 Nei M, Niimura Y, Nozawa M 2008. The evolution of animal chemosensory receptor gene  
713 repertoires: roles of chance and necessity. *Nature Reviews Genetics* 9: 951.
- 714 Nei M, Rooney A 2005. Concerted and birth-and-death evolution of multigene families.  
715 *Annual Review of Genetics* 39: 121–152.

- 716 Nevitt G 1999a. Foraging by seabirds on an olfactory landscape. *American Scientist* 87: 46–  
717 53.
- 718 Nevitt G 2000. Olfactory foraging by Antarctic procellariiform seabirds: life at high  
719 Reynolds numbers. *The Biological Bulletin* 198: 245–253.
- 720 Nevitt G 1999b. Olfactory foraging in Antarctic seabirds: a species-specific attraction to krill  
721 odors. *Marine Ecology Progress Series* 177: 235–241.
- 722 Nevitt G, Haberman K 2003. Behavioral attraction of Leach's storm-petrels (*Oceanodroma*  
723 *leucorhoa*) to dimethyl sulfide. *Journal of Experimental Biology* 206: 1497–1501.
- 724 Nevitt G, Losekoot M, Weimerskirch H 2008. Evidence for olfactory search in wandering  
725 albatross, *Diomedea exulans*. *Proceedings of the National Academy of Sciences* 105:  
726 4576–4581.
- 727 Nevitt G, Reid K, Trathan P 2004. Testing olfactory foraging strategies in an Antarctic  
728 seabird assemblage. *Journal of Experimental Biology* 207: 3537–3544.
- 729 Nevitt G, Veit R, Kareiva P 1995. Dimethyl sulphide as a foraging cue for Antarctic  
730 procellariiform seabirds. *Nature* 376: 680.
- 731 Niimura Y 2012. Olfactory receptor multigene family in vertebrates: from the viewpoint of  
732 evolutionary genomics. *Current Genomics* 13: 103–114.
- 733 Niimura Y 2009. On the origin and evolution of vertebrate olfactory receptor genes:  
734 comparative genome analysis among 23 chordate species. *Genome Biology and Evolution*  
735 1: 34–44.
- 736 Niimura Y, Matsui A, Touhara K 2014. Extreme expansion of the olfactory receptor gene  
737 repertoire in African elephants and evolutionary dynamics of orthologous gene groups in  
738 13 placental mammals. *Genome Research* 24: 1485–1496.
- 739 Niimura Y, Nei M 2005. Evolutionary dynamics of olfactory receptor genes in fishes and  
740 tetrapods. *Proceedings of the National Academy of Sciences* 102: 6039–6044.

- 741 O'Dwyer T, Ackerman A, Nevitt G 2008. Examining the development of individual  
742 recognition in a burrow-nesting procellariiform, the Leach's storm-petrel. *Journal of*  
743 *Experimental Biology* 211: 337–340.
- 744 Organ C, Rasmussen M, Baldwin M, Kellis M, Edwards S. 2010. Phylogenomic approach to  
745 the evolutionary dynamics of gene duplication in birds: Wiley & Sons, New York.
- 746 Oxley J 1999. Nesting distribution and abundance of Leach's storm-petrel (*Oceanodroma*  
747 *leucorhoa*) on Bon Portage Island, Nova Scotia. [Acadia University, Wolfville, Canada.
- 748 Pearce D, Hoover B, Jennings S, Nevitt G, Docherty K 2017. Morphological and genetic  
749 factors shape the microbiome of a seabird species (*Oceanodroma leucorhoa*) more than  
750 environmental and social factors. *Microbiome* 5: 146.
- 751 Pluznick J, et al. 2009. Functional expression of the olfactory signaling system in the kidney.  
752 *Proceedings of the National Academy of Sciences* 106: 2059–2064.
- 753 Pond K, Posada D, Gravenor M, Woelk C, Frost S 2006. GARD: a genetic algorithm for  
754 recombination detection. *Bioinformatics* 22: 3096–3098.
- 755 Pond S, Muse S. 2005. HyPhy: hypothesis testing using phylogenies: Springer, New York,  
756 NY.
- 757 Quignon P, et al. 2005. The dog and rat olfactory receptor repertoires. *Genome Biology* 6:  
758 R83.
- 759 Quinlan A, Hall I 2010. BEDTools: a flexible suite of utilities for comparing genomic  
760 features. *Bioinformatics* 26: 841–842.
- 761 Restrepo D, Lin W, Salcedo E, Yamazaki K, Beauchamp G 2006. Odortypes and MHC  
762 peptides: Complementary chemosignals of MHC haplotype? *Trends in Neurosciences* 29:  
763 604–609.
- 764 Rodríguez-Ruano S, et al. 2015. The hoopoe's uropygial gland hosts a bacterial community  
765 influenced by the living conditions of the bird. *PLoS One* 10: e0139734.

- 766 Roper T 1999. Olfaction in birds. *Advances in the Study of Behavior* 28: 247.
- 767 Saito H, Chi Q, Zhuang H, Matsunami H, Mainland J 2009. Odor coding by a mammalian  
768 receptor repertoire. *Science signaling* 2: ra9.
- 769 Santoro S, Dulac C 2012. The activity-dependent histone variant H2BE modulates the life  
770 span of olfactory neurons. *Elife* 1: e00070.
- 771 Seutin G, White BN, Boag PT 1991. Preservation of avian blood and tissue samples for DNA  
772 analyses. *Canadian Journal of Zoology* 69: 82–90.
- 773 Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM 2015. BUSCO:  
774 assessing genome assembly and annotation completeness with single-copy orthologs.  
775 *Bioinformatics* 31: 3210–3212.
- 776 Sin YW, Buesching CD, Burke T, Macdonald DW 2012. Molecular characterization of the  
777 microbial communities in the subcaudal gland secretion of the European badger (*Meles  
778 meles*). *FEMS Microbiology Ecology* 81: 648–659. doi: doi: 10.1111/j.1574-  
779 6941.2012.01396.x
- 780 Smit A, Hubley R, Green P 2015. RepeatMasker Open-4.0. 2013–2015. Available at  
781 [www.repeatmasker.org](http://www.repeatmasker.org).
- 782 Spehr M, et al. 2003. Identification of a testicular odorant receptor mediating human sperm  
783 chemotaxis. *Science* 299: 2054–2058.
- 784 Stamatakis A 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of  
785 large phylogenies. *Bioinformatics* 30: 1312–1313.
- 786 Steiger S, Fidler A, Kempnaers B 2009a. Evidence for increased olfactory receptor gene  
787 repertoire size in two nocturnal bird species with well-developed olfactory ability. *BMC  
788 Evolutionary Biology* 9: 117.

- 789 Steiger S, Fidler A, Valcu M, Kempenaers B 2008. Avian olfactory receptor gene repertoires:  
790 evidence for a well-developed sense of smell in birds? *Proceedings of the Royal Society*  
791 *B: Biological Sciences* 275: 2309–2317.
- 792 Steiger S, Kuryshv V, Stensmyr M, Kempenaers B, Mueller J 2009b. A comparison of  
793 reptilian and avian olfactory receptor gene repertoires: species-specific expansion of group  
794  $\gamma$  genes in birds. *BMC Genomics* 10: 446.
- 795 Sudmant P, et al. 2010. Diversity of human copy number variation and multicopy genes.  
796 *Science* 330: 641–646.
- 797 Tamura K, et al. 2011. MEGA5: molecular evolutionary genetics analysis using maximum  
798 likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology*  
799 *and Evolution* 28: 2731–2739.
- 800 Theis K, et al. 2013. Symbiotic bacteria appear to mediate hyena social odors. *Proceedings of*  
801 *the National Academy of Sciences* 110: 19832–19837.
- 802 van-Buskirk R, Nevitt G 2008. The influence of developmental environment on the evolution  
803 of olfactory foraging behaviour in procellariiform seabirds. *Journal of Evolutionary*  
804 *Biology* 21: 67–76.
- 805 van-der-Linden C, Jakob S, Gupta P, Dulac C, Santoro S 2018. Sex separation induces  
806 differences in the olfactory sensory receptor repertoires of male and female mice. *Nature*  
807 *Communications* 9: 5081.
- 808 Vandewege M, et al. 2016. Contrasting patterns of evolutionary diversification in the  
809 olfactory repertoires of reptile and bird genomes. *Genome Biology and Evolution* 8: 470–  
810 480.
- 811 Warham J. 1990. *The Petrels. Their Ecology and Breeding Systems*. London: Academic  
812 Press.

813 Whittaker D, et al. 2016. Social environment has a primary influence on the microbial and  
814 odor profiles of a chemically signaling songbird. *Frontiers in Ecology and Evolution* 4: 90.  
815 Wyatt TD. 2003. *Pheromones and animal behaviour: communication by smell and taste*.  
816 Cambridge: Cambridge University Press.  
817 Zelano B, Edwards S 2002. An MHC component to kin recognition and mate choice in birds:  
818 predictions, progress, and prospects. *The American Naturalist* 160: S225–S237.  
819 Zomer S, et al. 2009. Consensus multivariate methods in gas chromatography mass  
820 spectrometry and denaturing gradient gel electrophoresis: MHC-congenic and other strains  
821 of mice can be classified according to the profiles of volatiles and microflora in their  
822 scent-marks. *Analyst* 134: 114–123.

823

824

#### 825 **Author contributions**

826 S.Y.W.S, G.N. and S.V.E. designed research; S.Y.W.S. performed research; S.Y.W.S.  
827 and A. C. analyzed data; S.Y.W.S. wrote the paper and all authors contributed to revised  
828 versions.

829

#### 830 **Acknowledgements**

831 This research was supported by NSF (award numbers: NSF Grant IOS-1258784, NSF  
832 IOS 0922640/IBN 0212467 and NSF Grant IOS 1258828). We thank Lee Adams and David  
833 Shutler for logistical support, Marcel Losekoot for data management, Brian Hoover and  
834 Logan Lewis-Mummert for field assistance at UC Davis, Prof. Shelley Adamo and Laura  
835 Hall at Dalhousie University and the Bauer Core Facility at Harvard University (especially  
836 Jennifer Couget, Christian Daly and Claire Reardon) for laboratory assistance. We thank Tim  
837 Sackton for his help with genome assembly. The computations in this paper were performed

838 on the Odyssey cluster at Harvard University and supported by Harvard University Research

839 Computing.

840

841 **Data Accessibility**

842 The draft genome and transcriptomic data are available via Dryad (DOI will be provided

843 later).

844

845 The authors declare no conflict of interest.

846

## Figures

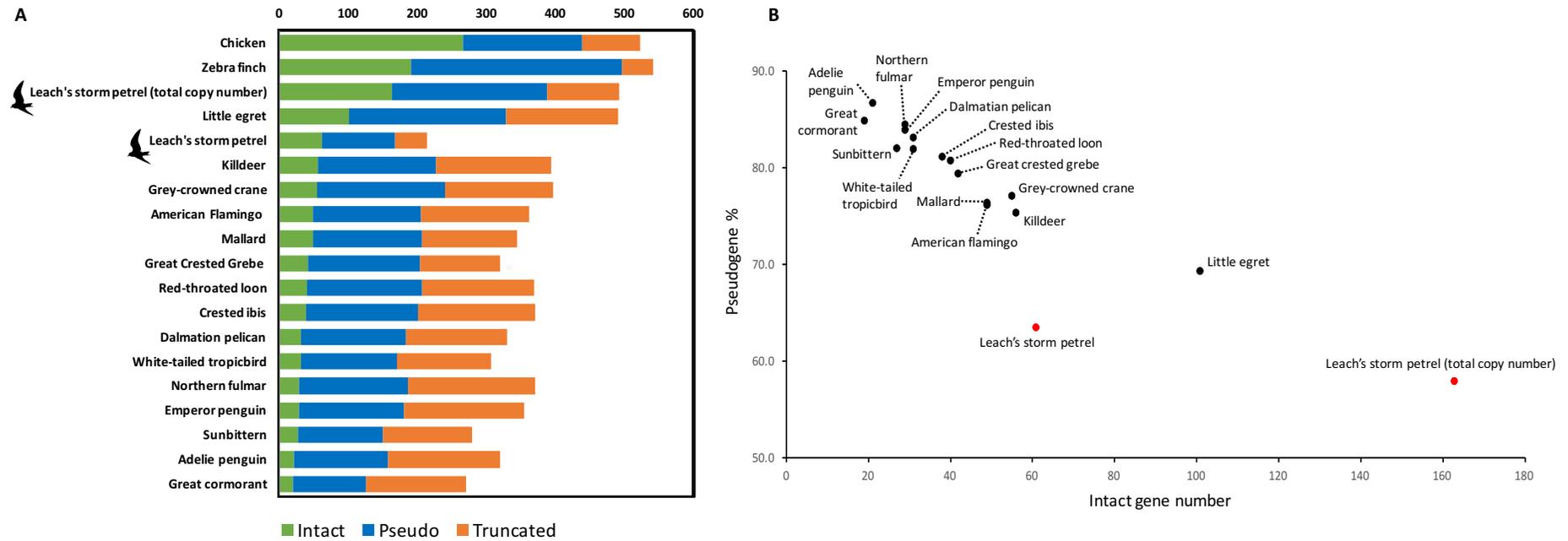
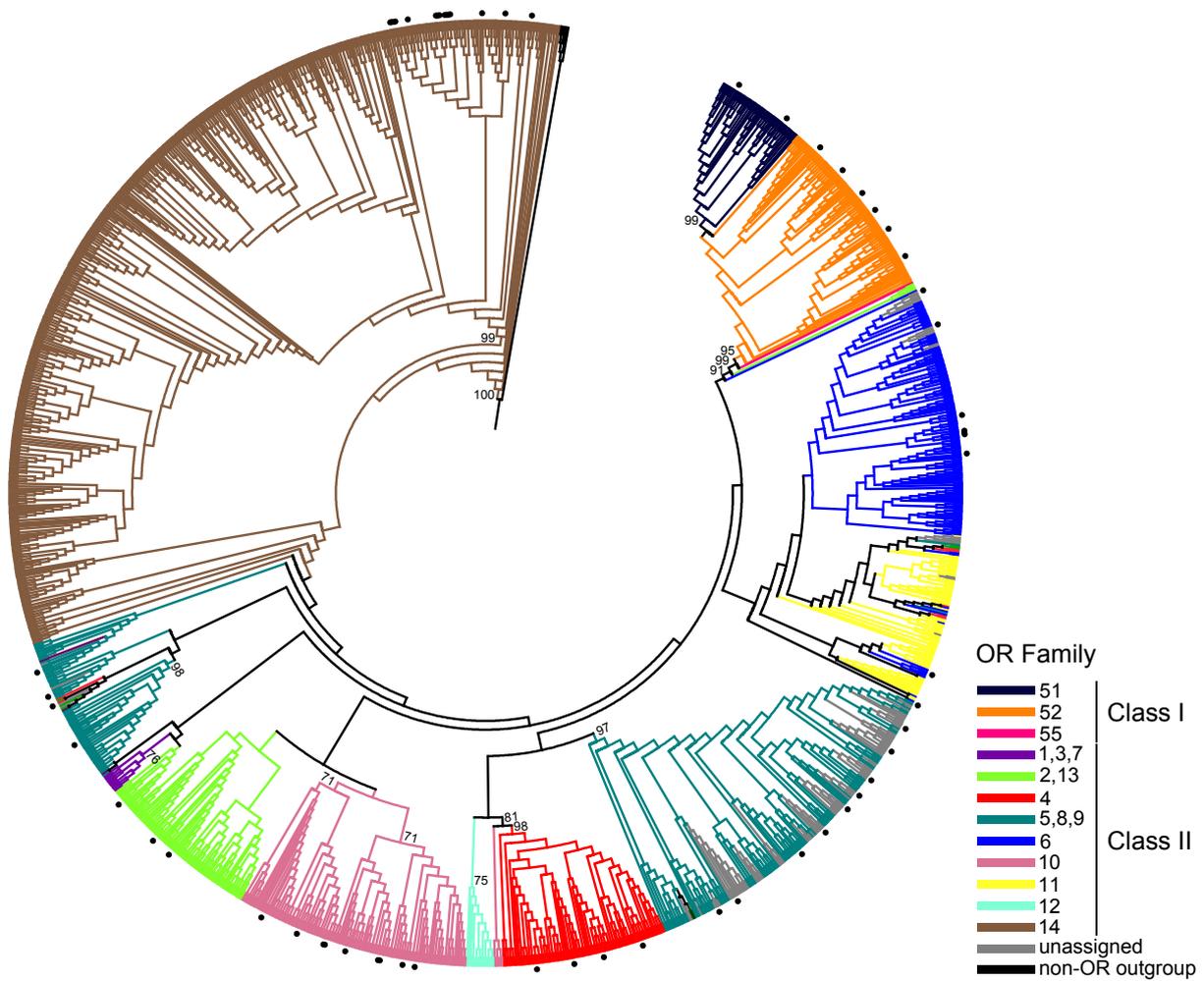
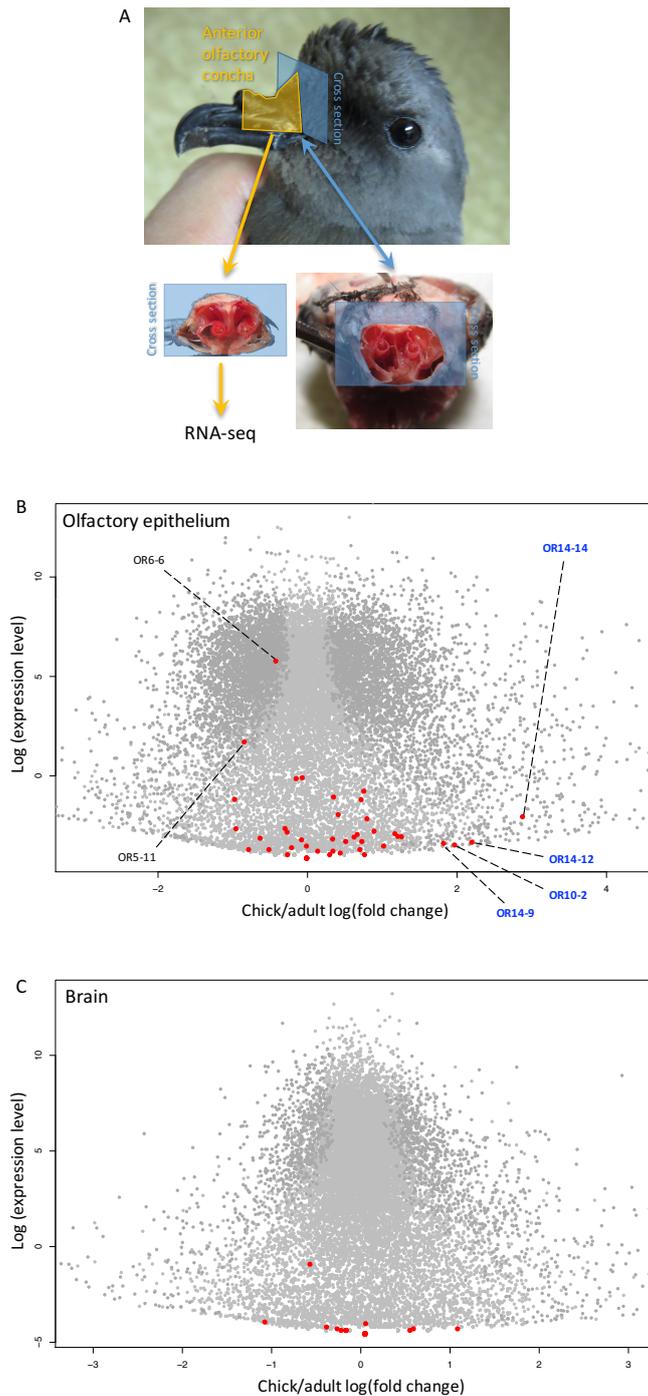


Figure 1 A) The number of truncated, pseudo-, and intact OR genes in waterbirds, chicken, and zebra finch. B) The number of intact genes plotted against the percentage of pseudogenes within the same genome in waterbirds. Both the OR gene number estimations based on genome annotation and copy number calculation in the Leach's storm petrel are shown. The numbers for all species except the Leach's storm-petrel are from Khan *et al.* (2015).

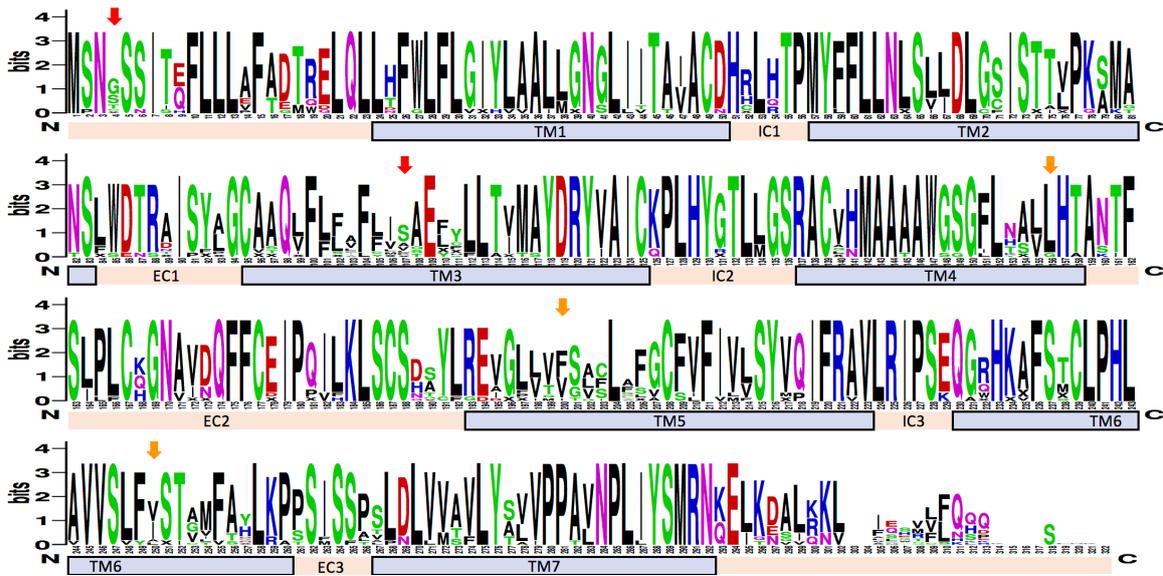


**Figure 2** Maximum-likelihood topology of relationships among intact ORs. Branches for individual OR sequences are coloured according to OR family, and branch lengths are not drawn to scale. Circle symbols indicate intact OR genes identified in Leach's storm petrel. Percentage support values from 500 bootstrap replicates are indicated for major clades with > 70% support.



**Figure 3** OR genes expression in the olfactory epithelium of anterior olfactory concha of the Leach's storm petrel. A) Anterior olfactory concha of the Leach's storm petrel for RNA-seq. B) Differentiation expression of the genes in chick versus adult olfactory epithelium. Differentially expressed genes are in dark grey. OR genes are highlighted in red. Four OR genes with higher expression in chicks are labelled with their names in blue. Two most

highly expressed OR genes are also labelled. C) Differentiation expression of the genes in chick versus adult brain. No OR genes were differentially expressed in the brain.



**Figure 4** Amino acid sequence variation of the intact family 14 OR genes in the Leach's storm petrel. Red and orange arrows indicate significant positively selected sites identified by all and at least two methods, respectively. Locations of the transmembrane domains (TM1-7), intra-cellular domains (IC1-3), and extra-cellular domains (EC1-3) are shown. The overall height of the stack of symbols indicates the sequence conservation at that codon position. The height of amino acid symbols with the stack indicates the relative frequency of each amino acid at that codon position. Numbers below the stacks indicate codon position.

## Tables

**Table 1** Assembly statistics for Leach's storm petrel genome

	Leach's storm petrel genome
Estimated genome size	1.24 Gb
%GC content	42.1
Total depth of coverage	80x
Total contig length (bp)	1,181,786,487
Total scaffold length (bp, gapped)	1,195,165,757
Number of contigs	17396
Contig N50 (bp)	165.4 kb
Number of scaffolds	1697
Scaffold N50 (with gaps)	8.7 Mb
Total BUSCOs	4817/4915 (98.0%)
Complete BUSCOs	4654/4915 (94.7%)

**Table 2** The number of intact, pseudo-, truncated, and fragment OR genes and their average coverage in the Leach’s storm petrel genome.

	Number of genes	Total copy number <sup>A</sup>	Average coverage
<b>Intact</b>	<b>61</b>	<b>163</b>	<b>2.7</b>
<b>Truncated</b>	<b>20</b>	<b>51</b>	<b>2.6</b>
<b>Total pseudogene</b>	<b>106</b>	<b>224</b>	<b>2.1</b>
• Pseudogene	• 45	• 81	
• pseudogene/fragment	• 49	• 103	
• pseudogene/fragment/truncated	• 2	• 4	
• pseudogene/truncated	• 10	• 36	
<b>Total fragment</b>	<b>27</b>	<b>54</b>	<b>2</b>
• fragment	• 24	• 48	
• fragment/truncated	• 3	• 6	
<b>Total (I+T+P+F)</b>	<b>214</b>	<b>492</b>	<b>2.3</b>

<sup>A</sup> Refer to the Discussion for the limitation of copy number estimation.

**Table 3** Positively selected sites detected by five approaches, along with integrated analysis, in genes of OR family 14 in the Leach’s storm petrel. The sites detected by more than two methods are in bold and underlined.

No. of sequences	Positively selected sites				
	SLAC	FEL	MEME	FUBAR	Integrative
15	<b><u>4, 107</u></b>	<b><u>4</u></b> , 38, 99, <b><u>107</u></b> , 110, 134, <b><u>156</u></b> , <b><u>200, 250</u></b>	<b><u>4</u></b> , 6, 25, 47, 93, <b><u>107</u></b> , 154, <b><u>156</u></b> , 172, 182, 183, <b><u>200</u></b> , 203, 238, <b><u>250</u></b> , 254, 261, 306, 307, 311, 312	<b><u>107</u></b>	<b><u>4</u></b> , 6, 25, 38, 47, 93, 99, <b><u>107</u></b> , 110, 134, 154, <b><u>156</u></b> , 172, 182, 183, <b><u>200</u></b> , 203, 238, <b><u>250</u></b> , 254, 261, 306, 307, 311, 312