

1 **Title**

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

3

4 **Authors**

5 Simon Yung Wa Sin^{1,2,4*}, Alison Cloutier^{1,4}, Gabrielle Nevitt³, and Scott V. Edwards¹

6

7 **Author affiliation**

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

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

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

14 ⁴ 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

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 α -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 γ -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 [rrectablePEfastq.py](https://github.com/harvardinformatics/TranscriptomeAssemblyTools/blob/master/FilterUncorrectablePEfastq.py)). 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 GOzilla 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) to infer potential recombination breakpoints and estimate ω . 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) 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 (γ -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 ω 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.
- 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 γ 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

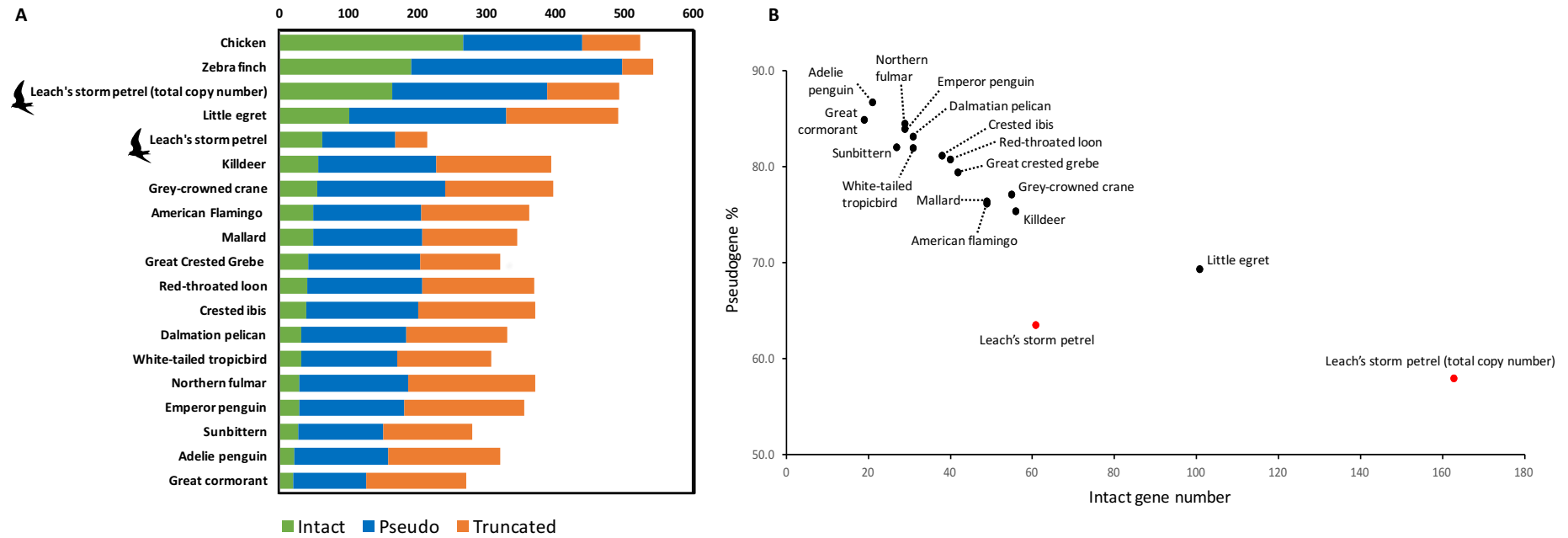


Figure 1 A) The number of truncated, pseudo-, and intact OR genes in waterbirds, chicken, and zebra finch. B) The number of intact genes plotted against the percentage of pseudogenes within the same genome in waterbirds. Both the OR gene number estimations based on genome annotation and copy number calculation 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).

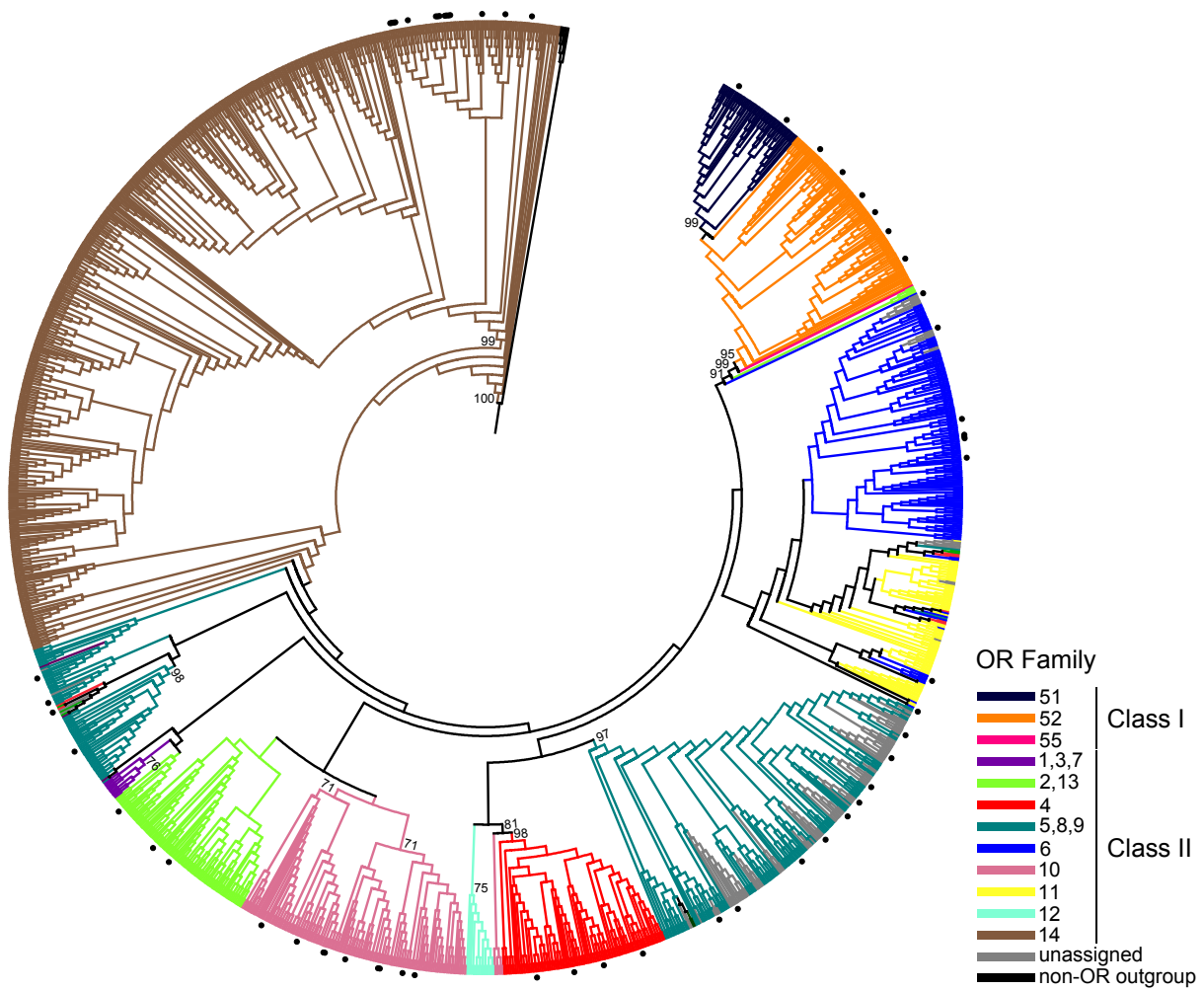


Figure 2 Maximum-likelihood topology of relationships among intact ORs. Branches for individual OR sequences are coloured according to OR family, and branch lengths are not drawn to scale. Circle symbols indicate intact OR genes identified in Leach's storm petrel. Percentage support values from 500 bootstrap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

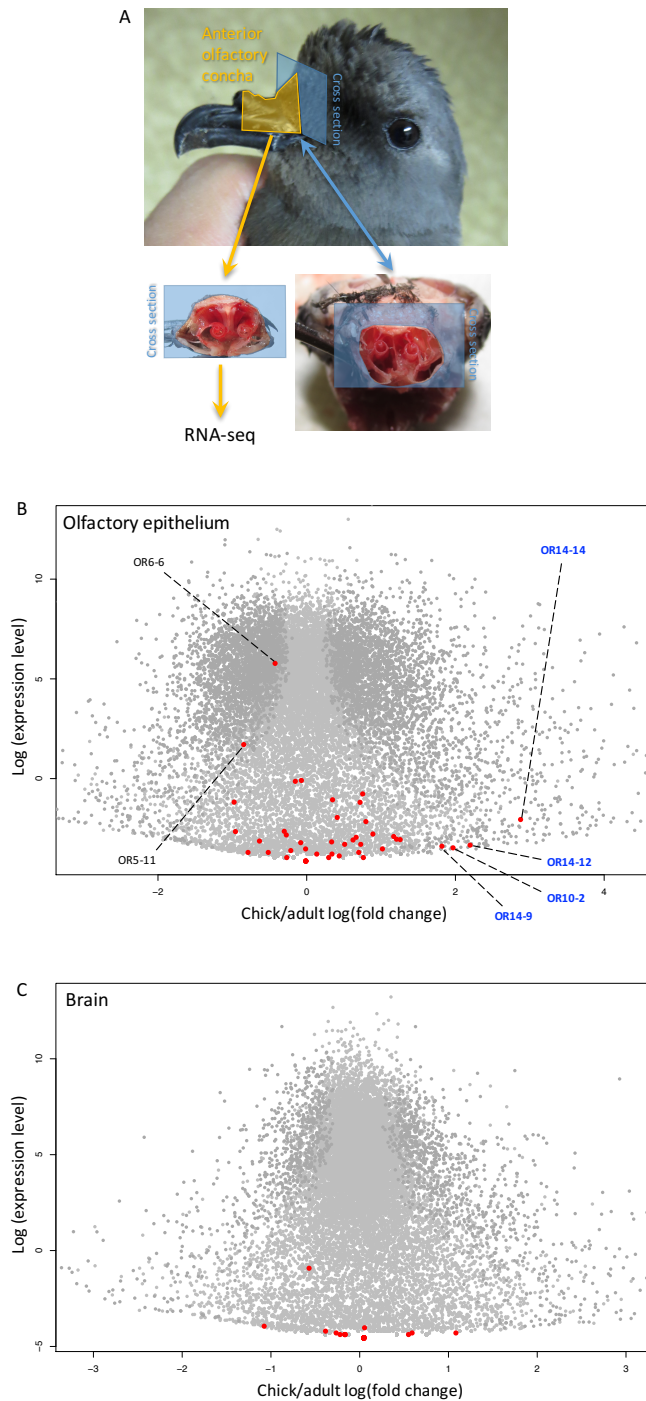


Figure 3 OR genes expression in the olfactory epithelium of anterior olfactory concha of the Leach's storm petrel. A) Anterior olfactory concha of the Leach's storm petrel for RNA-seq. B) Differentiation expression of the genes in chick versus adult olfactory epithelium. Differentially expressed genes 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.

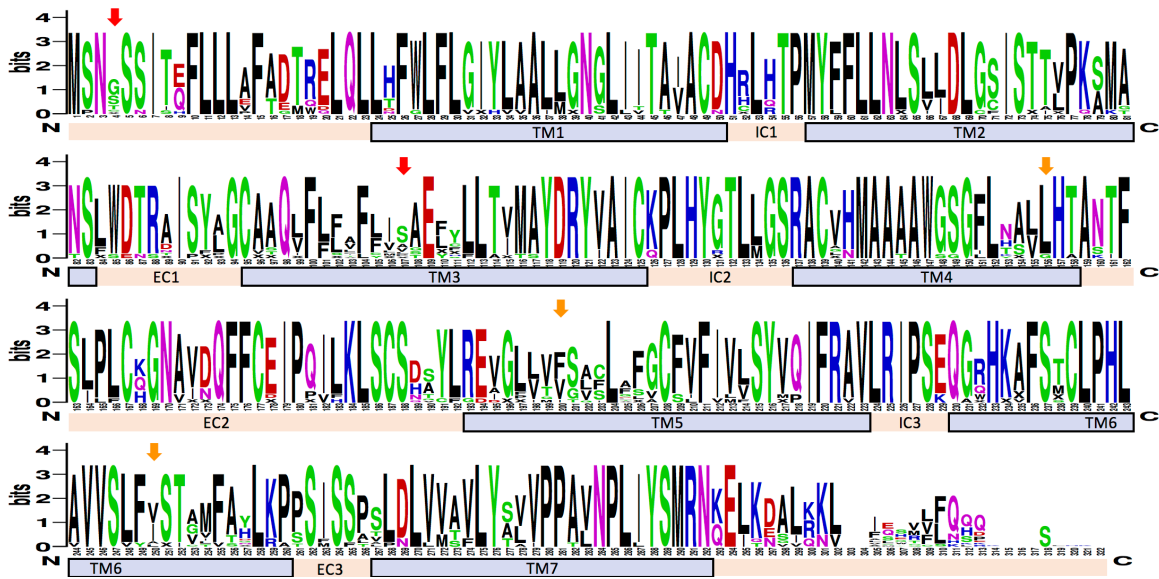


Figure 4 Amino acid sequence variation of the intact family 14 OR genes in the Leach's storm petrel. Red and orange arrows indicate significant positively selected sites identified by all and at least two methods, respectively. Locations of the transmembrane domains (TM1-7), intra-cellular domains (IC1-3), 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 ^A	Average coverage
Intact	61	163	2.7
Truncated	20	51	2.6
Total pseudogene	106	224	2.1
• Pseudogene	• 45	• 81	
• pseudogene/fragment	• 49	• 103	
• pseudogene/fragment/truncated	• 2	• 4	
• pseudogene/truncated	• 10	• 36	
Total fragment	27	54	2
• fragment	• 24	• 48	
• fragment/truncated	• 3	• 6	
Total (I+T+P+F)	214	492	2.3

^A 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	<u>4, 107</u>	<u>4</u> , 38, 99, <u>107</u> , 110, 134, <u>156</u> , <u>200, 250</u>	<u>4</u> , 6, 25, 47, 93, <u>107</u> , 154, <u>156</u> , 172, 182, 183, <u>200</u> , 203, 238, <u>250</u> , 254, 261, 306, 307, 311, 312	<u>107</u>	<u>4</u> , 6, 25, 38, 47, 93, 99, <u>107</u> , 110, 134, 154, <u>156</u> , 172, 182, 183, <u>200</u> , 203, 238, <u>250</u> , 254, 261, 306, 307, 311, 312