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1 **Reed warblers in the Marquesas Islands: song divergence and plumage convergence of two distinct**
2 **lineages**

3

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12

13 **Abstract**

14 The Pacific Ocean is the site of the most important diversification of insular reed warblers
15 (*Acrocephalus*). In the Marquesas Islands (Eastern Polynesia), reed warblers belong to two distinct
16 lineages that have reached the archipelago independently. We used mitochondrial sequences and
17 microsatellite markers and found evidence of gene flow between the two lineages. One island in
18 particular, Nuku Hiva, seems to act as the main contact zone between the two taxa. We then used
19 reflectance spectrometry to evaluate the variation of carotenoid colouration among all populations.
20 The Marquesan reed warblers exhibit convergent evolution in carotenoid-based plumage
21 colouration, but variation among islands was important. Having two different phylogenetic origins,
22 the two lineages acquired their yellow colour independently and there is stochastic variation in

23 plumage among islands. Finally, we analysed the songs of reed warblers from the main islands. The
24 multifactorial analysis showed that vocalizations of the Marquesan reed warblers differ slightly
25 between species, significant differences between islands leading to different dialects. Vocal
26 characteristics may act as the main pre-zygotic barriers when birds from different lineages come into
27 contact. This study provides another example of convergence in plumage evolution in insular bird
28 species. Furthermore, it highlights the importance of integrative analyses, using both genetic and
29 phenotypical characters, for the study of the evolutionary history of the Pacific Ocean avifauna.

30

31 **Keywords**

32 Acrocephalidae, plumage colouration, convergent evolution, vocalization, island evolution,
33 microsatellites, mtDNA, Oceania.

34

35 **Introduction**

36 The Acrocephalidae represent a morphologically conserved family within the Old World warblers
37 (Fregin *et al.* 2009; Alström *et al.* 2013). Most species have nondescript plumage, with dull brownish
38 or greyish upperparts and whitish to bright yellow underparts, a few species showing supercilium,
39 crown stripe, or streaks (Kennerley and Pearson 2010). Having 6 genera and 61 species (Gill and
40 Donsker 2018), they reach their highest species richness in western central Asia and Africa. Reed
41 warblers forage primarily on insects and in the temperate regions most reed warblers are migratory.
42 Around one-third of the species are found on oceanic islands, often very far away from continental
43 land masses, where they have become sedentary endemics. On most of these tropical or subtropical
44 islands, acrocephalids occupy completely different habitats from those occupied by their continental
45 relatives. Although wetlands are used when available, most species are found in woodland, dry bush
46 or scrub (Leisler and Schulze-Hagen 2011; Bell 2018).

47 Having 20 endemic species, the Pacific Ocean represents the most important diversification of insular
48 acrocephalids, all in the genus *Acrocephalus*. They have a disjunct distribution, as they are found
49 discontinuously from Micronesia to the Pitcairn group and Hawaii (only small islands with shrubland),
50 but are absent from the large and species-rich islands in southern Melanesia and central Polynesia
51 (for instance Vanuatu, Fiji, Samoa). The Pacific reed warblers are able to thrive on very small and
52 isolated islands, where resources are sparse, an ability that is consistent with part of the supertramp
53 definition (Diamond 1974). They are often the only land bird on low-lying atolls. However, they also
54 have notably long lifespans and are territorial, living in pairs or trios (Mosher 2006; Thibault and
55 Cibois 2017).

56 Molecular phylogenies provide evidence for a complex pattern of colonization of the Pacific by reed
57 warblers, in which most lineages are much younger than the ages of the islands they occupy (Fregin
58 *et al.* 2009; Cibois *et al.* 2011). The Pacific reed warblers are divided into two main clades
59 (supplemental Figure S1). The first includes all species from Micronesia (except Guam), Australia
60 (colonized from an insular ancestor, thus a case of reverse colonization), and two Polynesian taxa
61 from the Line Islands and the southern Marquesas. The second lineage includes all remaining Eastern
62 Polynesian taxa and the Hawaiian species. The taxa endemic to three archipelagos (Mariana,
63 Marquesas and Society Islands) are polyphyletic, suggesting several independent colonization events
64 (Cibois *et al.* 2007; Cibois *et al.* 2008; Saitoh *et al.* 2012). Overall, the colonization of reed-warblers in
65 the Pacific has been very rapid within the last two million years and it did not follow a regular,
66 stepping-stone-like pattern, as distribution solely might have suggested.

67 Leisler and Winkler (2015) analysed the morphological variation among insular and continental
68 species and found general evidence of convergent evolution of stronger legs, shorter rectal bristles
69 and broader and rounder wings on islands compared to continental taxa. Their results contrast with
70 those of Komdeur *et al.* (2004), who found no flight differences between the Seychelles Warbler
71 (*Acrocephalus sechellensis*) and three species that are migratory but do not migrate long-distances.

72 They concluded that the Seychelles Warbler may have a behavioural reluctance to cross open sea-
73 water rather than a morphological impediment. It is likely that the conclusions of these two different
74 studies are both true within the large insular reed warbler radiation: the original colonizers must
75 have good capacities to sustain over-water dispersal, even if those were facilitated by rare events like
76 hurricanes. But during this oceanic radiation, the reed warblers have also developed behavioural
77 adaptations to their new local environment: often a wider prey spectrum, a more terrestrial niche, or
78 a different social system, and this has led to many idiosyncrasies that often differ between
79 archipelagos. Thus, global rules, as proposed when studying the evolution of insular birds, often do
80 not fit the evolution of reed warblers in the Pacific.

81 The most puzzling example of complex colonization pattern is found in the Marquesas Islands, which
82 have two distinct lineages, one in the north and another in the south (Cibois *et al.* 2007). The
83 Northern Marquesan Reed Warbler (*Acrocephalus percernis*) is closely related to Polynesian species
84 from the Tuamotu and Gambier Islands, whereas the Southern Marquesan Reed Warbler
85 (*Acrocephalus mendanae*) is closely related to Micronesian taxa (from Nauru and Pagan). Despite
86 their distinct origin, both reached the Marquesas at roughly the same time, around 0.6 million years
87 ago (Mya) according to a mitochondrial molecular clock. The two taxa are cryptic in the sense that
88 they were considered a single species on the basis of plumage similarities - both are relatively large
89 birds, with bright yellow underparts, olive upperparts (Kennerley and Pearson 2010). Furthermore,
90 that this archipelago is so remote led Murphy and Mathews (1928) and all subsequent ornithologists
91 to assume they would have been monophyletic.

92 In addition to the molecular phylogeny, Cibois *et al.* (2007) studied the morphology of these two
93 species using multivariate analyses. Overall there is a large overlap in morphometrics, although on
94 average the southern taxon is larger than the northern one. The analyses also suggested a higher
95 influence of resource availability than of phylogeny on the morphology of the Marquesan reed
96 warblers: birds on dry and smaller islands tend to be smaller than birds on large islands, irrespective

97 of their phylogenetic origin. These first genetic and morphological studies, however, left unresolved
98 several issues regarding the evolution of these two taxa. First, the molecular phylogeny was based
99 solely on mitochondrial genes and a limited number of individuals (26, covering all subspecies and 2
100 to 5 individuals per island). Our first objectives were then to increase the sampling and to include a
101 combination of mitochondrial and nuclear markers. As trials with nuclear intron sequences revealed
102 no variation between these two recently diverged groups, we chose hypervariable microsatellite
103 markers that have been previously used to decipher the phylogeography of insular populations of
104 reed warblers (Richardson *et al.* 2000; Cibois *et al.* 2010). Second, while previous ornithologists have
105 noted plumage in these groups as being similar, qualitative comparisons performed by human
106 observers can in some cases mislead (Eaton and Johnson 2007). To investigate the colouration of the
107 two Marquesas reed warblers in a more objective way, we used reflectance spectrometry to evaluate
108 the variation of carotenoid colouration among all populations. Finally, we measured and compared
109 vocalizations, which are known as major prezygotic barriers in many closely related passerine
110 species, in particular among species with similar plumages, like in *Acrocephalus* (Lemaire 1977) or
111 *Phylloscopus* (Martens *et al.* 2008). Marquesan reed warblers are known to produce a combination
112 of whistles and rasping sounds that includes many repetitions of particular motifs (Kennerley and
113 Pearson 2010). Subtle differences between the different subspecies have been noted, but not all
114 islands were sampled for sounds and individual variation was not assessed (MacPherson 1998).

115 Thus, our first goal in this study is to test whether gene flow occurs between the two taxa, and
116 additionally within each of the island groups. We then evaluate within this system two main
117 mechanisms known to play a role in bird species recognition, based on visual and vocal characters.

118 Globally insular birds are expected to have colour that is less elaborate than their continental
119 counterparts (Doutrelant *et al.* 2016), though in the Passeriformes there are many exceptions to this
120 rule. The Marquesan reed warblers have yellow underparts that are brighter than most continental
121 *Acrocephalus* (Bell 2018). This may be explained by convergent evolution, which occurs frequently in
122 insular species (Warren *et al.* 2005; Jones and Kennedy 2008). This convergence might have been

123 favoured by environmental factors over the signal divergence that is expected to be less important in
124 allopatric species than in sympatric taxa (Martin *et al.* 2010). Regarding vocalization, if gene flow
125 between islands is low, dialects are expected to develop over time by cultural drift or adaptation
126 (Potvin and Clegg 2015). But because the two Marquesan reed warblers have diverged relatively
127 recently, we might expect to identify differences in vocalization that could play a role in species
128 recognition, particularly if gene flow occurs between the two taxa.

129

130 **Methods**

131 ***Genetics***

132 From 2000 to 2010, we mist-netted and sampled 135 reed-warblers in the nine Marquesas islands
133 (supplemental Figure S1). We used playback of recorded songs to lure birds into nets. Because reed-
134 warbler males are strongly territorial, most birds captured were males (recognized as singing
135 individuals). We avoided recapture by marking the forehead of captured birds with biodegradable
136 paint and the locations of all singing individuals were geo-referenced. Additionally, we used toe pads
137 from 11 specimens collected by the Whitney South Sea Expedition in the American Museum of
138 Natural History (AMNH), in New York (for a summary of this expedition, see Thibault and Cibois
139 (2017)). These toe pads were sampled for a previous study (Cibois *et al.* 2007).

140 Genomic DNA was extracted, amplified and sequenced using standard protocols, as in Cibois *et al.*
141 (2007). We amplified and sequenced a portion of the mitochondrial cytochrome b gene with the
142 primers 43L/17H (879 bp) (Edwards *et al.* 1991; Cibois *et al.* 2007). Contigs were assembled using
143 Sequencher (Genecodes, Ann Arbor, Michigan). All sequences were deposited in GenBank (accession
144 numbers MK517292-MK517409; Table S1). For the mitochondrial data, number of haplotypes,
145 nucleotide diversity and mismatch distributions were calculated using DnaSP 6.12.01 (Rozas *et al.*
146 2017). Haplotype networks were constructed using Network 5.0.0.3 (Bandelt *et al.* 1999). Signals of

147 population expansion were tested using Harpending's raggedness index r (Schneider and Excoffier
148 1999) using Arlequin 3.1 (Excoffier *et al.* 2005). We also used Fu's F_s and Tajima's D to test for
149 selective neutrality and population equilibrium (Tajima 1989) using DnaSP. The Θ_{ST} values between
150 pairs of population were calculated in Arlequin (sequential Bonferroni corrections were applied when
151 appropriate). For the calculation of the pairwise permutations, the individuals from Hatutaa were
152 included into the Eiao population, and the individuals sampled in Mohotani were included into the
153 Hiva Oa group.

154 We genotyped six microsatellite loci originally developed for the Seychelles Warbler: Ase12, Ase13,
155 Ase18, Ase34, Ase51, Ase55 (Richardson *et al.* 2000). Microsatellite fragments were amplified using a
156 touch-down PCR as described in the original publication. Genotyping was conducted on a 310
157 Genetic Analyzer (Applied Biosystem) using multiplex PCR (Qiagen). Results were visualized using
158 Genescan Analysis 3.1.2 (Applied Biosystems). The genetic diversity was quantified for allelic
159 richness, observed heterozygosity and expected heterozygosity. These statistics were estimated
160 using Arlequin, Fstat 2.9.3.2 (Goudet 1995) and MSA (Dieringer and Schötterer 2003). Deviations
161 from Hardy-Weinberg genotype frequency equilibrium were tested with Arlequin (10 000
162 permutations). The F_{ST} values between pairs of populations were calculated in Arlequin (sequential
163 Bonferroni corrections were applied when appropriate). For the calculation of the pairwise
164 permutations, the individuals from Hatutaa were included into the Eiao population, and the
165 individuals sampled in Mohotani were included into the Hiva Oa group.

166 Bayesian assignment of individuals to clusters without using prior information of the sampling
167 locations was conducted using Structure 2.3.1 (Pritchard *et al.* 2000) and the microsatellite data. The
168 inferred number of clusters, K , was tested from $K=1$ to $K=10$. Each run was pursued for 1 000 000
169 iterations, with a burnin period of 100 000. A correlated allele frequency and admixture model was
170 used. Convergence was assessed with ten independent runs. The inferred number of clusters K was
171 estimated using the value of K with the highest probability: posterior probabilities were computed as

172 indicated in Pritchard *et al.* (2000), and an additional ad hoc statistic (ΔK) was also estimated,
173 following the simulation study of Evanno *et al.* (2005). We estimated the number of immigrants per
174 generation using Migrate 3.6 (Beerli 2009), which follows a MCMC-based maximum-likelihood
175 approach based on an expansion of the coalescence model (from present to the most recent
176 common ancestor). The runs consisted of 10 short chains (sampling 10 000 trees) and three long
177 chains (sampling 100 000 trees) with a burn in period of 10 000 trees. All runs were repeated five
178 times to verify consistency of results. To avoid applying a unique mutation rate to the microsatellite
179 loci, we inferred the number of immigrants per generation N_i by multiplying the mutation-scaled
180 effective population size Θ to the mutation-scaled effective immigration rate M .

181 Finally, for both data sets, we tested the hypothesis of Isolation by distance (IBD) among island
182 groups using a Mantel test between the genetic distances (estimated as $\Theta_{ST}/(1 - \Theta_{ST})$ for mtDNA and
183 $F_{st}/(1 - F_{st})$ for microsatellites) and the logarithm of geographic distances (Rousset 1997).

184

185

186 ***Plumage colouration***

187 We assessed the carotenoid-based colouration of Marquesan reed warblers using reflectance
188 spectrometry, a method used to quantify colour independently of the spectral sensitivity of the birds
189 (Andersson and Prager 2006). We followed the techniques used in previous studies of plumage
190 colouration of several groups of Passeriformes (Hofmann *et al.* 2006; Kiere *et al.* 2007). Plumage
191 colour was measured using an Ocean Optics USB 2000 spectrometer, with a full spectrum Xenon light
192 source (Ocean Optics PX-2). Measurements were calibrated by a dark and a Spectralon diffuse white
193 standard (Labsphere, North Sutton, New Hampshire). We worked on specimens collected during the
194 Whitney South Sea Expedition and held at the American Museum Natural History collection. We
195 measured 9 to 15 museum skins per island (all males, not molting, in fresh plumage). We focused on

196 the bright yellow underparts of the birds; we measured three regions, throat, breast, and lower belly,
197 which correspond to the visible gradation from more intense on the throat to less intense on the
198 lower belly. The plumage regions followed the topography provided by Andersson and Prager (2006).
199 We took three non-overlapping measurements of each region, perpendicular to the plumage surface,
200 and we calculated the mean of the three measures. Initially we also measured the colour of the
201 nape. However, as preliminary analyses showed that the colour of this area was too patchy and gave
202 a large standard deviation between hue values, this region was removed from further analyses.

203 The evaluation of the colour space was based on three characters: hue, saturation and brightness.
204 Hue was estimated by the reflectance midpoint within the visible spectral range (400-700 nm, not
205 including the UV peak between 300 and 400 nm), which is a commonly used measure for carotenoid-
206 based plumage (Friedman *et al.* 2011). Saturation, or chroma, corresponds to our perception of the
207 richness of a hue. In the Marquesan reed warblers, the most pronounced colour is yellow (500-600
208 nm) and so we measured the saturation of this colour as the reflectance from 500-600 nm divided by
209 the overall reflectance (300-700 nm) and multiplied by 100 (i.e., yellow chroma, the percentage of
210 the feather's reflectance curve in the yellow part of the spectrum; Montgomerie 2006). Brightness
211 was measured as the mean reflectance over the spectral range (300-700 nm). We compared
212 Marquesan reed warblers with another bright yellow species, the Tahiti Reed Warbler (*Acrocephalus*
213 *caffer*). We tested the significance of variances between taxa and populations using Multivariate
214 analysis of variance (MANOVA and nested MANOVA, using the Pillai test for significance; Pillai 1955).
215 We used linear discriminant analysis (LDA), first to infer which of the colour variables we measured
216 were the most discriminant between populations. Second, we compared two discriminant functions:
217 (1) habitat richness in terms of vegetation diversity, in which the islands are classified either as "rich"
218 with more than 100 indigenous plants or "poor" with less than 100 plant species (due to its proximity
219 to the large Hiva Oa, Tahuata is classified as "rich" as well), and (2) phylogeny, in which the islands
220 are classified according to the two clades, Northern or Southern. All analyses were conducted using

221 the packages *ggplot2*, *Mass*, and *devtools* in R (R-Core-Team 2017), with the data standardized with
222 the *scale* function (zero mean and unit variance).

223

224 **Vocalization**

225 Between 1987 and 2012, we recorded males of each Marquesan population, using a Sony ECM 929LT
226 condenser microphone and a Sony WM D6C tape recorder. Tapes were digitized at the Muséum
227 national d’Histoire naturelle, Paris (MNHN, ISYEB) and sounds were deposited in the Sound Library of
228 the MNHN (Accession numbers MNHN-SO-2019-32 to MNHN-SO-2019-59). For the analysis, we
229 selected 4 males from each population (except Hatutaa and Mohotani). We used both short and long
230 songs, but not the alarm calls. Sonograms were generated and a song analysis was performed using
231 Avisoft-SASLab Pro 5.2.07 (Avisoft Bioacoustics). For each individual, up to 5 sequences of 4 seconds
232 continuous vocalization were selected. In our study, we separate two different components of the
233 songs, hereafter called syllables: the “whistle” (shown as horizontal or curved lines in sonograms, see
234 Figure 3) and the “rasping” (shown as multiple blurring lines in sonograms, see Figure 3). In each
235 sequence, we measured the number of syllables, the type of syllable (whistle or rasping), and the
236 number of time the whistle or rasping was repeated. For each syllable we measured duration, start
237 frequency, end frequency, peak frequency, maximum frequency, and minimum frequency (examples
238 for these measures can be seen on this webpage https://www.avisoft.com/tutorial_measure3.htm).
239 Mean values of these parameters over all sequences were computed for each individual. We tested
240 the significance of variances between taxa and populations using Multivariate analysis of variance
241 (MANOVA and nested MANOVA, using the Pillai test for significance), and linear discriminant analysis
242 to infer which of the variables were the most discriminant between populations. As in the analysis of
243 plumage colouration, we also compared two discriminant functions, “habitat richness” and
244 “phylogeny” (see above).

245

246 Results

247 Genetics

248 28 haplotypes were found in the mitochondrial sequences of over 143 individuals, global haplotype
249 diversity (Hd) and nucleotide diversity (Pi) being 0.92 and 0.34, respectively. The lowest genetic
250 diversity was found in Ua Pou (Hd 0.43 and Pi 0.01) and the highest in Nuku Hiva (Hd 0.72 and Pi
251 0.11). We compared haplotype diversity and island area and we found no significant relationship (R^2
252 = 0.2379, $p = 0.2669$, supplemental Figure S2). The haplotypes were divided into two main groups,
253 separated by 6 base pairs that corresponded to the two taxa. We found 15 haplotypes for *A.*
254 *percernis* (Hd 0.84, nucleotide diversity Pi 0.09, Figure 1a) and 13 haplotypes for *A. mendanae* (Hd
255 0.85, nucleotide diversity Pi 0.09, Figure 1b). A single individual from Nuku Hiva (*A. percernis*),
256 Nuku20, had a haplotype from the Southern group that was otherwise found only in individuals from
257 Ua Pou. This individual was identified as male in the field, and its sex was later confirmed with the
258 P2/P8 set of primers used for PCR-based sexing in birds (Griffiths *et al.* 1998). Mismatch distributions
259 for each taxon were multimodal (Figure 1), indicating historically stable populations, Fu's F_s (South
260 1.34, North -0.41) and Tajima's D (South 1.14, North 0.40) being non-significant. Harpending's
261 raggedness index was significant for South ($r=0.097$, $P<0.05$) and close to significant for North
262 ($r=0.074$, $P<0.10$), indicating a poor fit of the observed and expected mismatch distributions. Global
263 Θ_{ST} was high (0.69; 95% confidence interval 0.59-0.79), and pairwise Θ_{ST} indicated significant genetic
264 structure between all pairs of islands, except between two pairs of close islands (Hiva Oa-
265 Mohotani/Tahuata, Eiao-Hatutaa/Nuku Hiva). The Mantel test was significant for South ($p=0.0389$)
266 but not for North ($p=0.1696$), suggesting that some isolation by distance might be prevalent for the
267 mitochondrial haplotypes only in the Southern part of the archipelago (the most isolated islands, Ua
268 Pou and Fatu Iva, also have the most divergent sequences).

269 The allelic richness of the six microsatellite loci varied from 1.04 to 3.36 (Table S2). Deviation from
270 HWE ($\alpha = 0.05$) was found in two cases (Nuku Hiva for locus 1, Hiva Oa for Locus 3), but null allele

271 frequency estimated by FreeNa for these loci and populations were less than or equal to 0.2,
272 suggesting that null alleles were uncommon to rare in our dataset. Pairwise F_{ST} values averaged over
273 the six loci were high (0.28; 95% confidence interval 0.22-0.36), and these values indicated significant
274 genetic structure between all pairs of islands except two different pairs of islands (Fatu Iva/Ua Pou
275 and Hiva Oa-Mohotani /Tahuata). The Mantel test between genetic and geographic distances was
276 not significant for either of the two island groups ($p=0.5028$ and 0.4956 for South and North,
277 respectively). Bayesian clustering analysis revealed $K=4$ as the best estimate for the number of
278 clusters among Marquesan reed-warblers (Figure 2). The first cluster (in blue) corresponds mainly to
279 individuals from three northern islands (Eiao, Hatutaa, Nuku Hiva), the second cluster (in green)
280 corresponds to individuals from Ua Huka, the third (yellow) and fourth (red) correspond to a mixture
281 of individuals from the five southern islands (Fatu Iva, Hiva Oa, Mohotani, Tahuata and Ua Pou). The
282 two first clusters correspond mainly to *A. percernis*, the two others to *A. mendanae*.

283 Admixture was lowest in Ua Huka (100 % of individuals in cluster 2) and highest in Ua Pou (33% of the
284 individuals could not be attributed to a single cluster). Several individuals were classified in a cluster
285 that does not correspond to their taxon: six individuals from Nuku Hiva (*A. percernis*) were classified
286 in the yellow or red “Southern” clusters (but not Nuku20, the male with a mitochondrial haplotype
287 from Ua Pou) and five individuals from Fatu Iva and Mohotani (*A. mendanae*) were classified in the
288 green “Northern” cluster. Migrate results suggested gene flow between the two groups with a
289 migration rate larger from the Southern islands group ($m=6.64$) than on the opposite direction
290 ($m=2.44$).

291

292 ***Plumage colouration***

293 Colour measurements (means and standard deviations) are provided on Table S3. Values of hue,
294 chroma and brightness correspond to the general gradation observed on the underparts of all
295 individuals, from a more saturated yellow on the throat to less bright and less saturated yellow on

296 the lower belly. Overall the Tahiti Reed Warbler *A. caffer* is less yellow than the Marquesan reed
297 warblers: *A. caffer* and *A. percernis* differed significantly in the degree of saturation of yellow in the
298 breast (MANOVA's Pillai test $P < 0.001$) and in the throat ($P < 0.01$); *A. caffer* and *A. mendanae* also
299 differed in the degree of saturation of yellow in the breast ($P < 0.001$) and in the throat ($P < 0.01$).
300 Finally, they differed in the value of hue in the throat ($P < 0.01$) and lower belly ($P < 0.01$).

301 Within the Marquesan reed warblers, the colour measurements differed significantly when the two
302 species are compared and all variables considered ($P < 0.05$, Table 1), the only significant difference
303 being the hue of the throat ($P < 0.01$). However, the nested MANOVA revealed that the variation
304 among populations within taxa was more important than the variance between taxa, only six
305 variables having significant differences, including the hue of the throat (Table 1). It is likely that the
306 significance of this variable between taxa was due to the large variation observed among
307 populations. This assumption was confirmed by the results of the LDA, in which the confidence
308 ellipses of the two taxa overlapped almost completely in the projection of the two first axes
309 (supplemental Figure S3).

310 When the two taxa are analysed separately, the LDA coefficients revealed that in *A. mendanae*, the
311 saturation and the hue of the throat were the most discriminant factors in the first axis, and the
312 saturation and hue of the lower belly were the most discriminant factors for the second axis
313 (supplemental Figure S4). In this taxon, the population from Ua Pou was the most divergent, having
314 intense yellow in all its underparts (highest values of saturation, see supplemental Table S3), whereas
315 the birds from Fatu Iva exhibited the highest values of hue (throat, breast and lower belly).

316 In *A. percernis*, the hue of the breast and of the lower belly were the strongly explanatory factors in
317 the first axis, variables from the breast being important in the second axis (supplemental Figure S5).
318 The population on Hatutaa, the smallest and driest island of the archipelago, was the most divergent
319 in the LDA, having a light brownish yellow (high values for hue and low values for brightness, see
320 supplemental Table S3). Results of the LDA for the two different predictive functions were significant

321 ($P < 0.05$), but both functions misclassified almost one third of the individuals (27.19% individuals
322 misclassified with “Habitat richness”, and 25.43% with “Phylogeny”). This suggests that none of these
323 predictive functions described accurately the variation observed in the plumage colouration in the
324 Marquesan reed warblers.

325

326 ***Vocalizations***

327 Vocalization characteristics of the Marquesan reed warblers are given in supplemental Table S4. The
328 Marquesan reed warblers provide complex and diverse vocalizations, having 13 to 21 different
329 syllables within a 4 second interval. The repetition of similar motifs was found in all individuals,
330 although in different proportions. On average, individuals from Ua Pou used the highest number of
331 repetitions (3.6 repetitions during 4 seconds recording, see an example in supplemental Figure S6),
332 whereas the lowest numbers of repetitions (less than 1 during 4 seconds recording, on average) were
333 found for two individuals from Ua Huka and one bird from Tahuata. The ratio of whistle and rasping
334 syllables varied from 70% of whistle on Tahuata to 66% of rasping on Ua Huka. The vocalization
335 measurements differed significantly when the two species were compared and all variables
336 considered ($P < 0.001$, Table 2), significant difference being only for two variables, number of
337 repetitions and proportion of whistles (both with $P < 0.01$). The variation among populations within
338 taxa is also significant when all variables are considered ($P < 0.01$), and four variables were also
339 significant individually (number of syllables, number of repetitions, proportion of whistles, and
340 duration).

341 Overall, the two taxa differ in their vocalizations and there was little overlap in the LDA space, but
342 the variation between islands was substantial (Figure 3). The LDA coefficients revealed that the
343 proportion of whistles and the start frequency were the most discriminant factors in the first axis,
344 and the number of repetitions and the end frequency were the most discriminant factors for the
345 second axis. Results of the LDA for the two different predictive functions were significant ($P < 0.05$),

346 and both functions classified correctly almost all individuals, “Phylogeny” (3.6% of misclassified
347 individuals) being a better predictive function than “Habitat Richness” (7.1% misclassified
348 individuals). This suggests that despite the differences in song characteristics observed between
349 islands, the vocalizations of Marquesan reed warblers have kept patterns that correspond to each of
350 the two lineages.

351

352 **Discussion**

353 ***Gene flow between lineages***

354 Gene flow among avian species is not a rare phenomenon, particularly in passerines (Ottenburghs *et al.*
355 2015). This is even more apparent when studying closely related species, or groups of recently
356 diverged species, as gene flow often occurs during speciation (Nosil 2008; Mallet *et al.* 2016). For
357 instance, Reifová *et al.* (2016) showed that interspecific gene flow occurred during the *Acrocephalus*
358 radiation, and not only between sister species. Here we found evidence of gene flow between *A.*
359 *percernis* and *A. mendanae*, two species that inhabit distinct but close island groups in the Pacific
360 Ocean, with a different phylogenetic origin. One island in particular, Nuku Hiva, seems to act as the
361 main contact zone between the two taxa. The only individual with a conflicting mitochondrial
362 genotype (out of 143 sequences) was caught on this island, and six other individuals showed
363 evidence of admixture from Southern nuclear markers. This island is one of the largest in the
364 archipelago, with 380 km² and a summit at 1,227 m. Its reed warbler population is the largest today
365 in the Marquesas (Thibault and Cibois 2017). Its large coastline and high mountains might have
366 facilitated the arrival of birds from other islands. Interestingly, the asymmetry of gene flow between
367 the two taxa, more important from South to North than in the opposite direction, is consistent with
368 the current prevailing winds that blow from the southeast below the equator (Galzin *et al.* 2016).

369 In terms of plumage, birds from Nuku Hiva cannot be distinguished from the Eiao population
370 (supplemental Figure S5). The non-admixed individuals from Nuku Hiva are identical to the
371 Eiao/Hatutaa birds for nuclear markers (cluster blue in Figure 2); these three islands also share
372 mitochondrial haplotypes (Figure 1). But despite their genetic relatedness, the birds from Hatutaa
373 exhibit distinct plumage, having a light brownish yellow (high values for hue and low values for
374 brightness). On the other hand, the distinctiveness of birds from Ua Huka based on molecular
375 markers (original haplotypes in Figure 1 and the yellow cluster in Figure 2) was not reflected in
376 comparisons of their plumage (see their large overlap in the PCA space in supplemental Figure S5).
377 These examples highlight the overall discrepancy between genetic and plumage characters found in
378 the Marquesan reed warblers. Additionally, island size and distance between islands seemed to have
379 little influence on the observed genetic diversity.

380

381 ***Convergence in Plumage Colouration***

382 Both species have strong yellow colouration, especially in the underparts. *A. mendanae* does not
383 share this colour with its closest related species as it belongs to a group of brown reed warblers
384 ranging from Line Islands up to Australia and Saipan in the Mariana Islands (the “Micronesian Clade”
385 in Cibois *et al.* ‘s (2011) tree; see supplemental Figure S1). On the other hand, *A. percernis* belongs to
386 a group of mostly grey or brown species from the Cook Islands to Hawaii (the “Polynesian Clade”;
387 supplemental Figure S1) that includes also a few species with yellow colouration, mostly found in the
388 Society islands, the brightest being the Tahiti Reed Warbler. We showed that, in terms of plumage
389 colouration, the two Marquesan reed warblers are more similar to each other than either is to the
390 Tahiti Reed Warbler. Reflectance spectrometry measures demonstrate that they share similar
391 carotenoid-based plumage colouration, but local variation (i.e. among islands) is important. This
392 variation is not correlated to the habitat diversity of the islands. In contrast, size is influenced by
393 resource availability: reed warblers on dry and smaller islands tend to be smaller than birds on large

394 islands, irrespective of their phylogenetic origin (Cibois *et al.* 2007; Leisler and Winkler 2015). Having
395 two different phylogenetic origins, the two Marquesan reed warbler lineages acquired their yellow
396 colour independently but show stochastic variation in plumage among islands.

397 The evolution of convergent plumage colour often implicates selection, as suggested for melanistic
398 insular flycatchers (Uy and Vargas-Castro 2015; Uy *et al.* 2019) or in sexual dichromatism for *Petroica*
399 robins (Kearns *et al.* 2015). Many correlations between melanistic plumages and traits under
400 selection have been suggested, for instance aggressiveness (Ducrest *et al.* 2008), immunity (Gangoso
401 *et al.* 2011), or for the melanic form of the Tahiti Reed Warbler, camouflage in dark understories
402 (Cibois *et al.* 2012). Similarly, carotenoid compounds can be under selection during speciation
403 (mostly sexual selection for colouration) and they also have important roles in vision (von Lintig
404 2012), as antioxidants (Fiedor and Burda 2014), or in the immune system (Sepp *et al.* 2011).

405 Several hypotheses could thus be proposed to explain colour variation in the Marquesan reed
406 warblers system, involving the source of carotenoid, selection on the deposition of this pigment on
407 the plumage, or a combination of the two. The diet of the Marquesan reed warblers is known to be
408 rather eclectic, including mainly insects but also small vertebrates (some of them introduced), fruits
409 and seeds (Leisler and Schulze-Hagen 2011; pers. obs.). The level of endemism of insects and plants
410 is high in the archipelago (Lorence *et al.* 2016; Roderick and Gillespie 2016), so one can wonder
411 whether the “Marquesan yellow” pigment could be specific to an endemic prey or plant. An analysis
412 of debris found in feces might improve our knowledge of the diet of these birds (Ramage *et al.* in
413 prep.).

414 Native predators (raptors, mammals, snakes) are all absent from the archipelago, so predation is
415 probably not a leading factor in the Marquesan reed warbler system. Interspecific competition can
416 impact trait evolution such as by character displacement in closely related species (Pfennig and
417 Pfennig 2012). In the Marquesas Islands, competition might have been more important in the past
418 before the extinction of several terrestrial birds (Thibault and Cibois 2017). However, most of these

419 were parakeets and pigeons and their frugivorous diet would have only marginally overlapped with
420 that of the reed warblers. The monarch flycatchers are the only other native insectivorous passerines
421 in the Marquesas Islands. They are forest-dwelling birds, found in both dry and wet forests, whereas
422 reed warblers prefer today dry scrub habitats and avoid humid forests. However, we note that most
423 of the littoral and low altitude habitats have been highly modified in the archipelago, only relictual
424 native xerophyllous forests remaining (Lorence *et al.* 2016). It is difficult to evaluate how much space
425 was shared between monarchs and reed warblers in the past. Thus, we suggest that sexual selection
426 and pleiotropic effects, rather than predation and competition, have been the drivers of colour
427 divergence of these birds. Alternatively, plumage colour variation can be an adaptive response to
428 variation in light environments across habitats (McNaught and Owens 2002) and similar light
429 environments might have influenced the convergent plumage evolution of reed warblers in the
430 Marquesas. Analysis of whole genomes, however, would be necessary to explore the genetic basis
431 and the evolutionary processes that mediate convergent plumage colouration in the Marquesan reed
432 warblers, like recent studies on Holarctic redpoll finches (Mason and Taylor 2015) or Eurasian crows
433 (Poelstra *et al.* 2014).

434

435 **Vocalizations**

436 Fiedler (2011) showed that song characteristics in the Acrocephalidae are heavily influenced both by
437 phylogeny and natural history. For example, males of migratory species have larger repertoires than
438 tropical, sedentary species, and often include more mimicry. While a single species from the Pacific
439 Ocean was included in Fiedler's study (the Australian Reed Warbler *Acrocephalus australis*), Leisler
440 and Schulze-Hagen (2011) used her work, along with isolated observations, to infer that insular
441 species have in general simpler and shorter songs than continental species. Although examples of
442 simpler, almost call-like songs have been reported in two Polynesian species [Henderson Reed
443 Warbler *A. taiti*; (Graves 1992); and Rimatara Reed Warbler *A. rimitarae*; (Thibault and Cibois 2006)],

444 the global pattern is probably more complex and idiosyncratic. For instance, Bell's (2018) analysis of
445 numerous Pacific *Acrocephalus* showed that the mean frequency of song syllables was inversely
446 correlated with mean body size. He also suggested that the environment through which the sound is
447 transmitted also influences the song, lower frequencies being prevalent in forest and scrub habitats.

448 Although a comparison of the complexity of Marquesan reed warblers' songs with continental
449 species was beyond the objectives of this study, we noticed that the Marquesan reed warblers
450 provide long and complex vocalizations that include repetitions of similar motifs. The multifactorial
451 analysis showed that vocalizations of the Marquesan reed warblers differ slightly between species
452 and that significant differences between islands led to different dialects. However, these dialects
453 retained some common elements within each taxon, suggesting a phylogenetic component in the
454 vocalization of Marquesan reed warblers. Differences in habitat richness, as broadly defined by the
455 number of native plant species, were not significantly associated to their vocalization diversity.
456 Marquesan reed warblers live in open habitats, which should have similar abiotic acoustic properties.
457 This might have led to similar types of songs arising across the archipelago. In contrast, "continental"
458 species of reed warblers vocalize from inside dense vegetation such as reed beds. The variation in
459 their songs has been linked to differences in mating system and habitat quality (Leisler and Schulze-
460 Hagen 2011). In oscine passerines, males often learn singing behaviour from parental or
461 neighbouring tutors, and elements inherited in this fashion should favour the evolution of local
462 dialect (Wada 2010). In relatively small and isolated populations, the consequences of founder
463 effects and of vocal drift have also been shown to favour the creation of local dialects, sometimes
464 associated with a reduction of vocalization diversity compared to a larger source population
465 (Valderrama *et al.* 2013), but not always (Baker *et al.* 2006).

466

467 **Conclusion**

468 In summary, the Marquesan reed warblers, two taxa that originated from independent lineages in
469 the Pacific Ocean, exhibit convergent plumage colouration while retaining differences in songs. Vocal
470 characteristics may act as the main pre-zygotic barriers when birds from different lineages come into
471 contact. In a majority of passerines with a monogamous breeding strategy, dispersal is female-
472 mediated (Greenwood 1980), as shown in migratory species (Guerrini *et al.* 2014) and in at least one
473 insular situation (Paris *et al.* 2016). Although our data set was not designed to test sex-biased
474 dispersal within the Marquesas archipelago, the occurrence of mitochondrial haplotype introgression
475 is consistent with dispersal of at least one female between the two groups. Further work, in Nuku
476 Hiva in particular, might be necessary to unravel the mechanisms in action in the contact zone
477 between the two taxa. Until recently, mitochondrial DNA sequences and microsatellite loci (SSR,
478 simple sequence repeats) were the gold standards in population genetics. Now, the use of genomic
479 methods can provide hundreds or thousands of nuclear single-nucleotide polymorphisms (SNPs)
480 generated by restriction-site associated DNA sequencing (RAD-seq), leading to a broader evaluation
481 of the genome. While these new data set are not devoid of drawbacks, in particular related to
482 evolutionary noise (Mesak *et al.* 2014), the study of the Marquesan reed-warblers would benefit
483 from a genomic approach in the future.

484

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499

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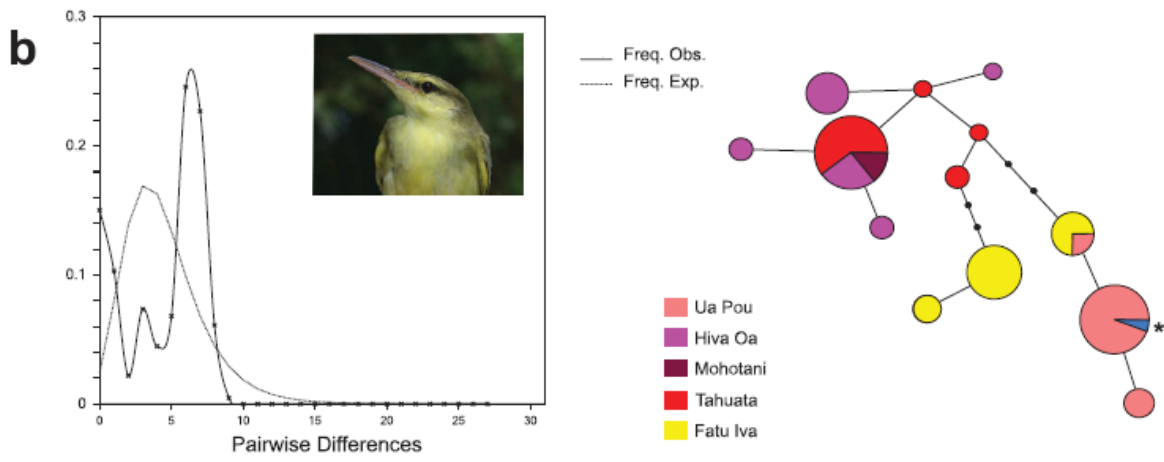
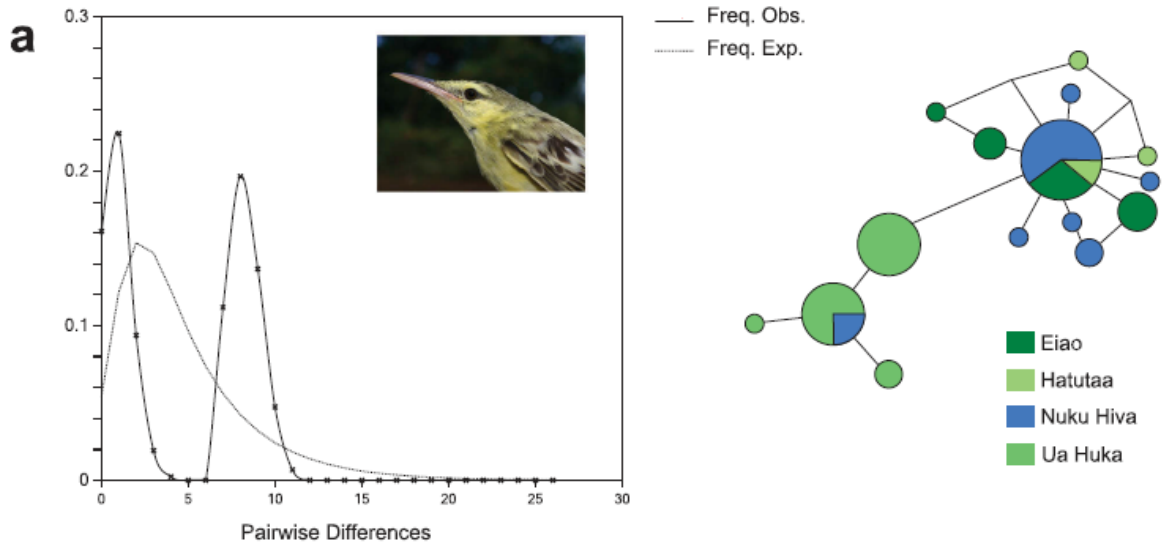
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677 Fig.1



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680 Fig.2

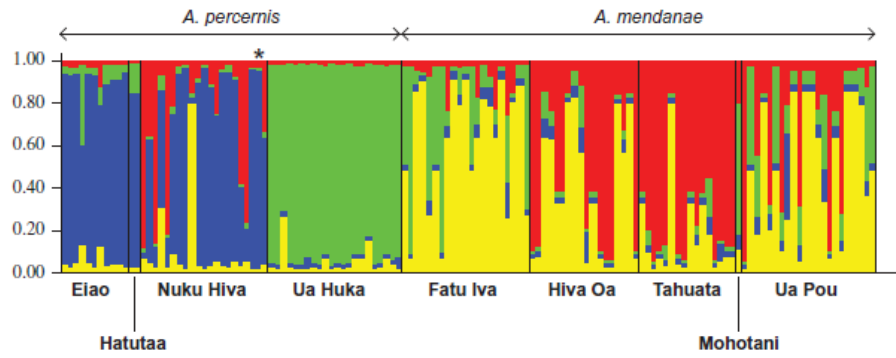
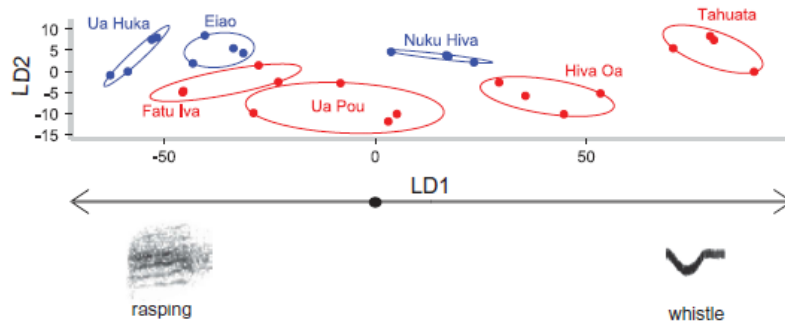


Figure 2. Individual barplots from each island corresponding to the optimal clusters ($K = 4$) found using an admixture model and the microsatellite loci in STRUCTURE (see online version for colour). The vertical lines each represent an individual, and colours represent the proportion of that individual assigned to each cluster. The asterisk indicates the individual sampled on Nuku Hiva (Nuku20) that presents a mitochondrial haplotype from the Southern group.



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