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1 # Evolutionary history of the Galápagos Rail revealed
2 # by ancient mitogenomes and modern samples

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23 **Abstract**

24 The biotas of the Galápagos Islands are probably one of the best studied island systems and have provided
25 a broad model of insular species' origins and evolution. Nevertheless, some Galápagos species remain
26 poorly characterized, such as the Galápagos Rail *Laterallus spilonota*. This bird species is one of the less
27 explored groups of endemic vertebrates on these islands, due to its elusive behavior, cryptic plumage and
28 restricted distribution. To date there is no genetic assessment of its origins and sister relationships to other
29 taxa, and importantly, there is no data on its current genetic diversity. This lack of information is critical
30 given the adverse fate of island rail species around the world in the recent past. Here we examine the
31 genetics of Galápagos Rails using a combination of mitogenome *de novo* assembly with multi-locus
32 sequencing (mtDNA+nuDNA) from both modern and historical samples. We show that the Galápagos
33 Rail is part of the 'American black rail clade', sister to Black Rail *L. jamaicensis*, with a colonization of
34 Galápagos dated to 1.2 Mya. The separate analysis of cyt*b*, ND2, and RAG-1 markers demonstrates
35 shallow population structure across sampled islands, possibly due to elevated island connectivity.
36 Additionally, birds sampled from Pinta possessed the lowest levels of genetic diversity, most likely
37 reflecting the impact of past bottlenecks due to habitat loss caused by invasive goats grazing on sensitive
38 habitat. The data presented here highlights the low genetic diversity in this endemic rail species and
39 suggests the use of genetic data (both modern and historical) to guide conservation efforts.
40

41 **Keywords:** ancient DNA; genetic diversity; island colonization; *Laterallus spilonota*; Rallidae;
42 phylogenetics

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43 **1. Introduction**

44 Studies of island biotas provide insights into the origin of species and the associated factors promoting it.
45 From characterizing morphological variation, documenting the time of divergence from mainland
46 counterparts, to ultimately understanding the formation of independently evolving lineages, insular species
47 have inspired scientists for centuries [1–6]. One such biological assemblage are species found on the
48 Galápagos archipelago. Generalities about Galápagos fauna have for example supported the hypothesis of
49 island colonization in relation to island age (i.e. progression rule) [7,8], provided text book examples of the
50 origin of adaptive radiations (i.e. Darwin's finches) [3,9], postulated the role of isolation in promoting
51 diversification (i.e. mockingbirds *Mimus* spp. [10], giant tortoises *Chelonoidis* spp. [11]), and reported
52 various origins for endemic species [12,13]. Most of these studies focus on highly charismatic species,
53 leaving almost no conspicuous species on the Galápagos unstudied. In particular, endemic vertebrate
54 species have been subject to extensive molecular and morphological assessment providing one of the
55 greatest insular biota datasets. The data has helped to raise awareness of conservation status and proposed
56 management recommendations for the preservation of several of these species. Unfortunately, though, a
57 few less charismatic endemic vertebrate species have suffered from a lack of such effort. This has not only
58 left a gap in our understanding of their evolutionary trajectory and origin, but most importantly, their
59 conservation status is poorly known and their extinction could potentially go unnoticed.

60 Species inhabiting oceanic islands are the most vulnerable to extinction due to human impact,
61 particularly those that are flightless. Flightlessness in birds has evolved as a consequence of the absence of
62 natural predators [14–16] creating opportunity for endemism. Rails (Rallidae) present high levels of
63 endemism, in many cases restricted to a single island [17,18]. Unfortunately, the introduction of predators
64 to such isolated regions make these species extremely susceptible to population declines and ultimately
65 extinction [19,20]. Historically, rails have succumbed quickly to human contact with the loss of as many
66 as 440–1,580 species on islands in the Pacific [21], where 22 of the 33 currently threatened rail species
67 (World Conservation Union) occur on islands, of which 86% are threatened by invasive mammals (BirdLife
68 International - IUCN databases).

69 The endemic Galápagos Rail *Laterallus spilonota* is an example of both this extreme adaptation to an
70 isolated system of oceanic islands and the fate of inconspicuousness, being the least studied land-bird species
71 on the Galápagos Islands. The historical distribution of Galápagos Rails has been documented by collectors
72 and naturalists since the early 1900s, allowing a reconstruction of the impact and decline of populations
73 compared to present data [22]. The introduction of rats and goats in the 18th century, by mariners and early
74 colonists using these islands for water and food supply [23–25], has had a direct impact on native species'
75 survival and ecosystem modification [26]. Galápagos Rails, once abundant as reported by Darwin (1896),
76 depend on the presence of wetlands and dense vegetation, but these habitats were decimated and eroded by
77 grazing goats and agricultural expansion, and altered by invasive plant species [27,28]. Likewise, rats and
78 cats have had a negative impact on the survival of rails given their inability to fly [29]. These events have
79 resulted in the extinction of several of the Galápagos Rail populations across the archipelago and a dramatic
80 decline in surviving populations [30,31]. Galápagos Rails are currently restricted to small pockets of natural
81 habitat in the highlands on five of the eight islands they historically inhabited. The species is currently listed
82 as Vulnerable (Red Data List)[32] and despite the eradication of goats since the 1970s and the ongoing pest
83 control efforts [33–35], rails still face continuous threats of habitat modification by invasive species [36]
84 and expansion of agriculture [37].

85 Galápagos Rails are one of the least studied land bird species on the Galápagos: i) there is no genetic
86 assessment of its phylogenetic relationships to other rails, ii) there are no estimations of the time since its
87 colonization, iii) no inference of its phylogeographic patterns and inter-island genetic relationships, and
88 importantly iv) there is no assessment of its current genetic diversity. This last effort is critical if we
89 acknowledge the adverse fate of rails around the world in the recent past and thus, the uncertainty of its
90 evolutionary potential in the future.

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91 Here, we focus on alleviating these aspects and bringing to light the evolutionary history of this
92 enigmatic endemic land bird of the Galápagos. We present data from a combination of fresh tissue samples
93 and century-old historic museum specimens collected by the California Academy of Sciences expedition to
94 the Galápagos in 1905–1906. The phylogenetic relationships for the Galápagos Rail proposed here for the
95 first time are based on high-throughput sequenced *de novo* assembly of its mitochondrial genome
96 (mitogenome), placing this species into a phylogenetic context. We also infer the timing of long-distance
97 colonization and focus on genetic diversity and relationships between islands.

98 **2. Materials and Methods**

99 *2.1. Study sites, sampling, and morphological data*

100 We focused the large-scale phylogenetic reconstruction on the generation of mitochondrial genetic data
101 from natural history collections. DNA extracted from toepad tissue allows mitochondrial genome assembly
102 at relatively low sequencing depth compared to DNA from nucleated avian blood cells, as the latter contain
103 significantly fewer mitochondria. While blood samples can be readily extracted from live birds, museum
104 specimens offered a better opportunity for destructive tissue sampling. Therefore, a series of Galápagos Rail
105 specimens deposited at the California Academy of Sciences (CAS) collected on Santa Cruz island in 1905–
106 1906 were accessed and toe pads sections from ten individuals were loaned. Additionally, modern samples
107 (blood samples) were obtained from several islands to complement the genetic assessment of Galápagos
108 Rails. The field work was carried out in four islands (May–July, 2017) and we concentrated our efforts to
109 the highlands (> 500 m). We sampled the islands of Santa Cruz, Santiago, Pinta, and Isabela. On each
110 locality we used playbacks to confirm the presence of rails as well as to define their territories. Birds were
111 captured using the V-netting with playback trapping method [38], which consists in the arrangement of
112 mist-nets forming a "V" and placed at ground level. Birds were lured inside the "V" using playback and led
113 into the mist-nets by two people that monitor and adjust dynamically to bird responses. From each
114 captured bird, we collected blood samples from the brachial vein in the field and blood was preserved on
115 FTA blood cards (Whatman®).

116 *2.2. Ancient DNA extraction and mitochondrial genomes*

117 Ancient DNA from museum samples (toe pads) was performed at UCLA's special ancient DNA facility
118 following phenol–chloroform extraction procedures, but with 30 ul DTT added to the initial incubation
119 step for the extraction from blood. DNA quantification was done using Epoch® (Bio Tek, USA) before
120 library preparations and amplification procedures. DNA quality was tested using Qubit. Whole-genome
121 next generation sequencing libraries with paired-end barcodes were prepared from five museum samples at
122 the UCLA Technology Center for Genomics and Bioinformatics (TCGB). The samples were sequenced
123 on the HiSeq3000 (150 PE) at the TCGB, yielding 2.13-2.37x10^7 shotgun sequencing reads per sample.
124 Read quality was determined using MultiQC [39]. There was little need for trimming, as reads were overall
125 considerably shorter than 150 bp due to fragmentation.
126 Exploration and preliminary mapping to Swinhoe's Rail *Coturnicops exquisitus* (Genbank accession
127 no. NC_012143) was performed for all samples in Geneious v. 10.2.6 [40] using their native mapper with
128 greatly relaxed settings, using 'custom sensitivity' only allowing mapping of full read pairs at minimum
129 mapping quality 7, maximum gaps per read 30%, maximum mismatches per read 35%, and maximum
130 ambiguity 10. We allowed up to 25 iterations, in which the first round reads are mapped directly onto the
131 reference, and the subsequent iterations onto the consensus of the previously mapping reads, as a way of
132 bridging regions of high divergence. Based on read quality, read length, and mapping success, we selected
133 the sample GR9 (ORN 274 CAS catalog number) for a reference assembly of *L. spilonota*. For GR9, the
134 mapping was complete in 5 iterations and 31,220 read pairs covered all of the *C. exquisitus* reference
135 sequence. We extracted these as *fastq* files, which we then used as mitochondrion-enriched starting material

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136 for a *de novo* assembly and annotation of the mitochondrial genome with MitoZ v. 2.4.α [41]. Using three
137 CPUs, we ran the *filter*, *assemble*, *findmitoscaf*, *annotate* and *visualize* modules with standard parameter
138 settings, specifying --clade Chordata, --insert-size 200 and --filter_taxa_method 3. The resulting annotated
139 genome was aligned with other rallid mitochondrial genomes and annotations were manually inspected
140 and adjusted with regard to the start of 16S rRNA, and the includion of an additional C in ND3 causing a
141 frameshift that is corrected through an unknown mechanism [42].
142 We mapped reads from the remaining samples to the circularized GR9 reference assembly in two steps
143 with the Geneious mapper. First we enriched for mitochondrial reads in a step using the setting 'low
144 sensitivity' with minimum mapping quality 15 and maximum mismatches per read 10% in two iterations.
145 We then took the mapping reads and re-mapped them with the 'highest sensitivity' setting allowing up to
146 25 iterations. Resulting contig consensus sequences were aligned with the GR9 reference, all annotations
147 lifted over, and every sequence variant position or ambiguity manually scrutinized against respective
148 mapping reads.

149 *2.3 Intraspecific genetics, genetic markers, and haplotype networks*

150 In addition to the high-throughput sequencing described above, we sequenced two mitochondrial and one
151 nuclear marker from a larger number of contemporary field samples. From each of the four visited islands
152 we sampled 60 individuals in total to provide more detailed population structure and intraspecific patterns
153 of diversification. Genomic DNA was extracted from blood cards using the Qiagen DNA Blood & Tissue
154 Kit and following the manufacturer's protocol. We focused on the mitochondrial genes cytochrome *b*
155 (cyt*b*) gene and nicotinamide dehydrogenase 2 (ND2), and the nuclear recombination-activating gene 1
156 (RAG-1). Amplification and sequencing of cyt*b* was done using primers L14990–H16065 and primers
157 L5143–H6313 for ND2 following the protocols described in Bonaccorso et al. [43], and for RAG-1, we
158 used primers R52–R53 as described by Johansson et al. [44] (Table S1). Gel electrophoresis was used to
159 confirm the presence and lenght of the amplifications, products were cleaned using ExoSAP-IT™ (Applied
160 Biosystems™, Massachusetss, USA) and sequenced by Macrogen. Returning sequences were aligned,
161 edited and trimmed using Geneious v. 10.2.6 [40]. Based on these sequences we represented intraspecific
162 relationships from each locus using minimum-spanning networks using the package *pegas* (Populations
163 and Evolutionary Genetics Analysis System) [45] as implemented in R 3.6.2 [46]. This package was also
164 used to estimate haplotypic (*Hb*) and nucleotide diversity (p) as a whole group for each locus, as well as
165 from individual islands with more than one haplotype.

166 *2.4. Interspecific phylogenetic reconstruction of mitogenomes and divergence time estimates*

167 2.4.1. Coding mitogenome dataset

168 We followed Stervander et al. [47] and chose to analyse all coding regions (CDS) of the mitochondrial
169 genome, partitioned per codon position (dataset "mtCDS"). As there was no appreciable phylogenetic
170 signal between Galápagos Rail mitogenomes, we arbitrarily selected a single sample, GR5 (ORN 262 CAS
171 catalog number), combined it through MAFFT alignent in Geneious to the dataset of Stervander et al. [47]
172 and new extant gruiform mitogenomes (for taxa and accession numbers, see Figure 1a), resulting in a matrix
173 comprising 32 species (22 species within Rallidae, 3 non-rallid species within Ralloidea, and 7 species within
174 Gruoidea) and 11,418 base pairs (bp).
175 We set up Bayesian Markov Chain Monte Carlo (MCMC) analyses for Beast v. 2.6.3 [48] partitioning
176 per codon position across all mitochondrial genes [47], each fitted with a general time-reversible (GTR)
177 model with four gamma categories (Γ) and an estimated proportion of invariant sites (I). We applied a
178 relaxed log-normal clock model, let speciation follow a birth–death prior, constrained Ralloidea as
179 monphyletic, and applied two priors for dating. Following Claramunt & Cracraft [49], we set a prior for
180 crown Gruiformes (i.e. the root of the tree) following an exponential distribution with rate parameter 8.5

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181 and offset 52 million years, that was fitted from the oldest fossils records from different parts of the world.
182 We used the fossil data compiled in Stervander et al. [47], setting a prior for Rallidae following a lognormal
183 distribution with mean 1.1, standard deviation 1.8, and offset 32.6 million years. However, as rightly
184 pointed out by Garcia et al. [50], the inclusion of *Belgirallus* to calibrate crown Rallidae may not be correct,
185 as *Belgirallus* may rather be representative of a stem rallid species [51]. We therefore primarily applied this
186 prior to date stem Rallidae (i.e. parental node of Rallidae, being the Ralloidea node, the most recent
187 common ancestor (MRCA) of Rallidae, Heliornithidae and Sarothruridae), and secondarily replicated the
188 analyses applying this prior to crown Rallidae (i.e. the MRCA of extant Rallidae species). The rate scaler
189 operators for A–C and C–T substitutions were modified (weight increased from 0.1 to 0.3) for improved
190 performance.

191 We ran three replicates of each analysis for 75×10^6 generations, sampled every 5×10^3 generations, of
192 which the first 5% were discarded as burn-in. Stationarity, high effectives sample sizes (ESS > 200), and
193 between-replicate convergence were observed for almost all parameters (with the exception of some
194 transition/transversion rate parameters for the second codon position, with ESS 135–200) in TRACER
195 v1.4 [52], and maximum clade credibility trees with divergence times for both stem and root were obtained
196 with median node heights calculated with TreeAnnotator [53]. Given convergence we used a single tree
197 per analysis, and drew them in R 3.6.2 [46] using the packages ape v. 5.3 [54,55] and Phytools v. 0.6-99
198 [56].

199 2.4.1. Two mitochondrial and one nuclear gene

200 In order to include more taxa, particularly focusing on the 'American black rail clade' *sensu* Stervander et
201 al. [47], we created a dataset "2mt1nc" based on the two mitochondrial genes cyt*b* (1,068 bp) and
202 cytochrome oxidase subunit I (COI; 747 bp), and the nuclear recombination activating gene 1 (RAG1; 930
203 bp). This dataset comprised 106 gruiform species, with varying degree of missing data. We followed the
204 substitution model evaluation and partitioning by Stervander et al. [47], setting up Beast analyses
205 partitioned by marker, with the substitution model HKY+Γ+I for and cyt*b* and COI, and K80+Γ+I for
206 RAG1. Priors for clock model, speciation model, and calibrations followed those for the mtCDS dataset,
207 as did operator modifications and run specifications, although number of generations 100×10^6.

208 **3. Results**

209 *3.1. Study sites and samples*

210 We captured a total of 60 individuals in the field, 15 from Santa Cruz, 16 from Isabela, nine from Pinta,
211 and 20 from Santiago. Out of the total number of samples from Santa Cruz, three were chicks, as were four
212 from Santiago. Rails were found at sites above 500 meters characterized by high coverage of bracken
213 (*Pteridium aquilinum*) and tall grass (*Pennisetum purpureum* and *Paspalum conjugatum*). Usually these
214 sites were in a matrix with native Galápagos Miconia *Miconia robinsoniana* and invasive quinine trees
215 *Cinchona pubescens* primarily in Santa Cruz, with guava trees *Psidium guajava* and blackberry (*Rubus* sp.)
216 in the rest of the islands sampled.

217 *3.2. Mitogenomes and genetic analysis*

218 Sequencing produced an average 4.64×10^7 ($4.44–4.99 \times 10^7$) reads per individual, with the average length
219 per samples 58–76 bp due to fragmentation (Table S2). The complete GR9 reference *de novo* assembly
220 produced by MitoZ was circularized and 17,045 bp long, with an average read depth of 61×. The GC
221 content was 42.4% and it contained 13 protein-coding genes, two rRNAs, 22 tRNAs, and a 1,526 bp long
222 control region (Figure S1). For the four remaining samples, the average number of reads per sample that
223 mapped to the GR9 reference assembly was 81.9–345.7 (Table S2). Three samples (in addition to the
224 reference GR9 also GR5 and GR8 (ORN 270 and 262 CAS catalog number respectively)) produced

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225 complete mitochondrial assemblies whereas two samples (GR2 and GR7; ORN 259 and 268 CAS catalog
226 number repectively) contained ≤0.1% missing data.
227 The five mitogenomes contained 27 variants, comprising 23 single nucleotide polymorphisms (SNPs)
228 and 4 insertion/deletion (indel) polymorphisms, distributed in protein-coding genes (15), control region
229 (11), and 16S rRNA (1; Table S3). Out of the 15 SNPs in protein-coding genes, 4 were in codon position
230 1 and 11 in codon position 3, with 12 being synonymous mutation and 3 non-synonymous (Table S3). All
231 variants were private to a single sample, with two exceptions: a synonymous SNP in COIII grouped GR7
232 and GR9 versus the three remaining samples, whereas an indel in the control region grouped GR9 and GR5
233 (reference haplotype), GR2 and GR8 (1 bp insertion), and GR7 (2 bp insertion; Table S3). Finally, there
234 was mononucleotide length variation in the beginning of 16S rRNA which was ambiguous and unresolved
235 due to low mapping success/coverage.

236 *3.3. Interspecific phylogenetic reconstruction and divergence time estimates*

237 The whole-mitochondrion phylogeny based on the mtCDS dataset, which did not include the Black Rail
238 *Laterallus jamaicensis* or the Dot-winged Crake *L. [Porzana] spiloptera*, recovered the Galápagos Rail as a
239 sister to the Inaccessible Island Rail *L. [Atlantisia] rogersi* with PP=1.0 (Figure 1a)
240 The mixed mitochondrial/nuclear marker phylogeny 2mt1nc, which contained more taxa but fewer
241 base pairs, recovered the Galápagos Rail as sister to the Black Rail, the common ancestor of which was the
242 sister of another sister species pair comprising the Inaccessible Island Rail and the Dot-winged Crake
243 (Figure 1b). All nodes received full support with PP=1.0, and the estimated median age of the MRCA of
244 the Galápagos Rail and the Black Rail was 1.1–1.2 Mya (95% HPDs 0.5–2.0 Mya), irrespective of whether
245 the Rallidae prior was applied to the stem or crown (Table 1). The MRCA of all four species was estimated
246 at a median age of 6.1 (stem) or 6.5 Mya (crown; for 95% HPDs, see Table 1), about a million years younger
247 than the ages estimated from the mtCDS dataset.
248 Overall, for both datasets, the placement of the Rallidae prior on the stem or crown had a small impact
249 (≤9%) on the dating of both younger and older nodes, including the MRCA of Rallidae (median age 31.5–
250 35.8 Mya; Table 1).

251 **Table 1.** Estimated ages for relevant nodes (most recent common ancestors, MRCA) in the
252 phylogenies created from the mtCDS and 2mt1nc sequence datasets, where the calibration density
253 for the age of Rallidae was placed on either the crown or stem of the family. Age estimates are
254 presented for the median and mean age as well as the 95% highest posterior density (HPD).

			Node age estimates		
Node (MRCA)	Sequence set	Rallidae prior	median	mean	95% HPD
L. spilonota/jamaicensis	2mt1nc	stem	1.1	1.2	0.5–2.0
		crown	1.2	1.2	0.6–2.0
L. spilonota/rogersi	2mt1nc	stem	6.1	6.2	3.8–8.8
		crown	6.5	6.6	4.3–9.0
	mtCDS	stem	7.5	7.6	4.8–10.5
		crown	7.3	7.4	4.9–10.1
Rallidae	2mt1nc	stem	31.5	31.8	25.1–39.2
		crown	33.6	34.3	32.6–38.0
	mtCDS	stem	36.1	36.4	30.3–43.1
		crown	34.6	35.3	32.6–40.0
Gruiformes	2mt1nc	stem	54.4	55.6	52.0–63.3
		crown	56.4	37.3	52.0–65.6
	mtCDS	stem	54.2	55.4	52.0–62.1
		crown	54	54.9	52.0–60.5

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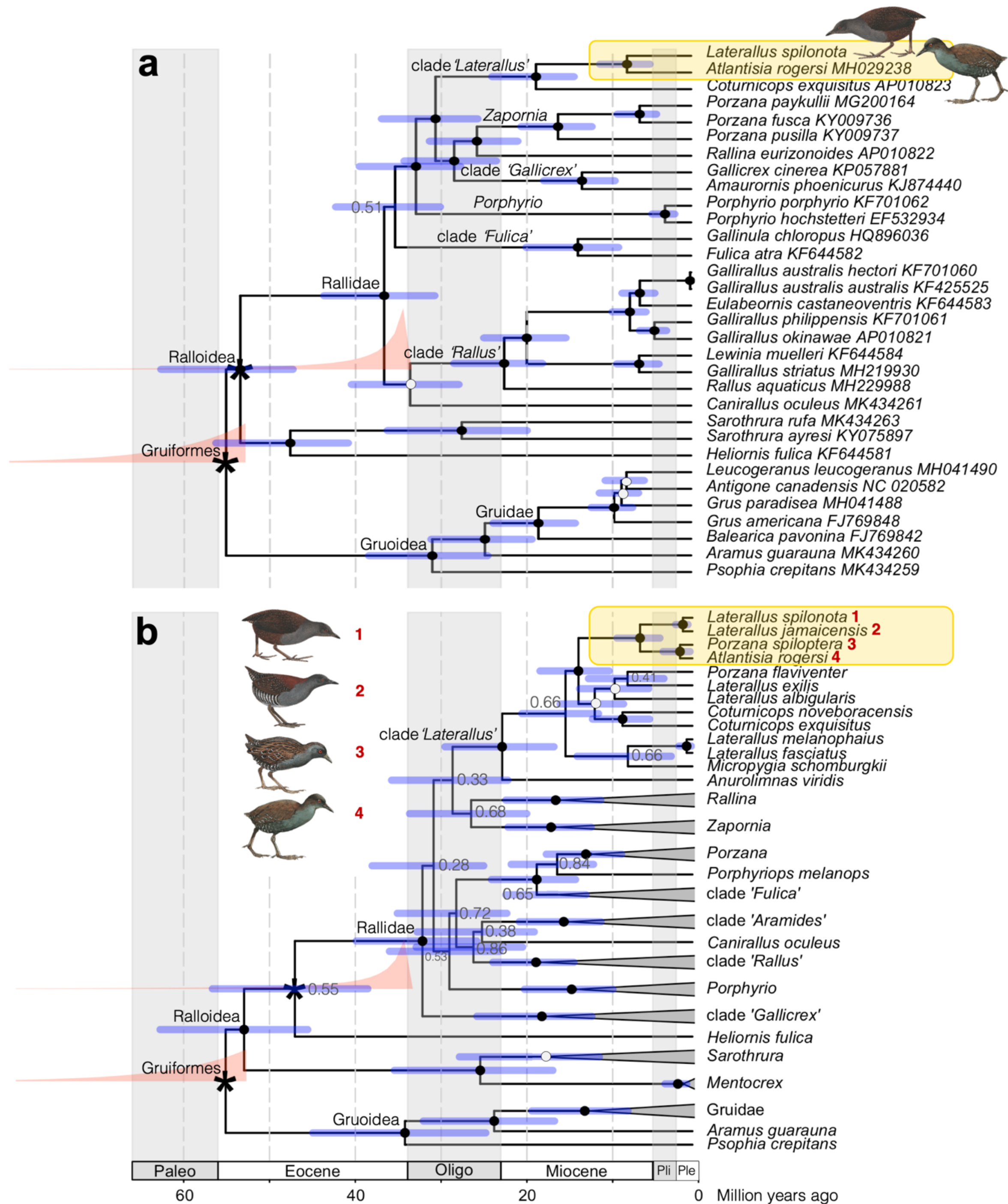

255

256 **Figure 1**. Dated phylogenies of family Rallidae showing the origin of the Galápagos Rail *Laterallus*
257 *spilonota*, based on **(a)** all coding sequences of mitochondrial genes (dataset mtCDS; GenBank
258 accession numbers stated at tips), and **(b)** the mitochondrial genes cytochrome *b* and cytochrome
259 oxidase I and coding sequence of the nuclear recombination-activating gene 1 (dataset 2mt1nc; for
260 GenBank accession numbers and taxon selection, see Data Accessibility). The chronograms are based
261 on a relaxed clock model with fossil-based calibrations of stem Rallidae and crown Gruiformes. We
262 also ran alternative analyses in which the Rallidae calibration was applied to the crown node, with
263 yielding small differences of estimated node ages (see Table 2). Nodes that were time-calibrated are
264 indicated with an asterisk, and the prior densities are drawn in salmon. Posterior probabilities (PP)
265 are represented by filled circles (PP = 1.0), open circles (0.94 ≤ PP ≤ 0.99), or stated at nodes if lower.
266 Node are drawn at median ages, with the 95% highest posterior density represented with blue

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bars. Shading represent geological periods—Paleogene (Paleo); Eocene; Oligocene (Oligo)—and epochs of the Neogene period: Miocene; Pliocene (Pli); Pleistocene (Ple). Every ten million years is indicated with dashed vertical lines. Illustrations reproduced with permission from Lynx Edicions ©.

3.4. Intraspecific genetics, genetic markers, and haplotype networks

We obtained 59 sequences for cyt*b* (720 bp), 51 sequences for ND2 (969 bp), and 58 sequences for RAG-1 (302 bp). Overall genetic diversity (*Hb*) values were 0.439 for cyt*b*, 0.580 for ND2 and 0.034 for RAG-1. We report overall nucleotide diversity (p) of 0.0006 for cyt*b*, 0.0007 for ND2 and 0.0001 for RAG-1. Values for each island for both *Hb* and p are reported in Table 2, with Santa Cruz possessing the highest values for both *Hb* (0.791) and p for ND2 compared to the other islands and markers. Similarly, Santa Cruz and Santiago presented the highest values for both genetic metrics for cyt*b*. Pinta was the island that contained populations with the lowest values for all markers. RAG-1, being a nuclear marker, presented the lowest values across islands. Correspondingly, haplotype networks reported high levels of haplotype sharing across islands with shallow differences within haplotypes for each marker (Figure 2). We recovered three haplotypes for cyt*b*, all separated by 1 bp, shared across all islands; five haplotypes for ND2 comprising 4 bp difference, with no private haplotype at any given island; and two haplotypes for RAG-1 with 1 bp difference, one of the haplotypes private to Santiago. Cyt*b* haplotype I (LS02_Cytb) presented the highest frequency (n=43) and was found on all four islands, followed by haplotype II (LS07_Cytb; n=9) found on Santiago, Santa Cruz and Isabela, and haplotype III (LS06_Cytb; n=7) found on Santiago and Santa Cruz. ND2 haplotype I (LS05_ND2) had the highest frequency (n=32) and was found on all four islands, haplotype II (LS06_ND2; n=7) found only on Santiago and Santa Cruz, haplotype III (LS21_ND2; n=5) found only on Isabela, haplotype IV (LS03_ND2; n=4) found on Santiago and Santa Cruz, and finally haplotype V (LS07_ND2; n=3) found only on Santa Cruz. RAG-1 haplotype I (LS02_RAG1) presented the highest frequency (n=57) and was found on all four islands, whereas haplotype II (LS58_RAG1) was found in only one individual on Santiago (n=1).

4. Discussion

We found that the endemic Galápagos Rail forms a monophyletic group indicative of a single colonization event to the Galápagos Islands around 1.2 Mya, with negligible differences depending on whether the *Belgirallus* fossil was considered a crown or stem rallid for the calibration of the dated tree (Table 1), an overall pattern contrasting the findings of García et al. [50]. In comparison with other Galápagos land bird colonizers, rails coincide with the estimated arrival of Darwin's finches (1.5–1.0 Mya [57,58] and flycatchers (genus *Pyrocephalus*: 1 Mya [59]; *Myiarchus*: 850 kya [60]). This also suggests that ancestors of the Galápagos Rail arrived to older islands first, most likely Cristóbal and Santa Cruz (~5 and 3 million years old respectively), moving west and colonizing new islands as these were formed by volcanic activity (Isabela ~0.5 Mya and Fernandina ~0.4 Mya, which it currently inhabits). Interestingly, speciation events have been idiosyncratic and variable in groups of the same time of origin. Finches have diversified into over 18 species [57] and *Pyrocephalus* flycatchers into two [59] compared to a single taxa of Galápagos Flycatcher *Myiarchus magnirostris* and Galápagos Rail. This phenomenon could be explained by an intrinsic evolvability in some groups compared to others, as explained by Chaves et al. [61] for Darwin's finches and Hawaiian honeycreepers compared to other insular taxa of the same age, and sympatric to the same island systems.

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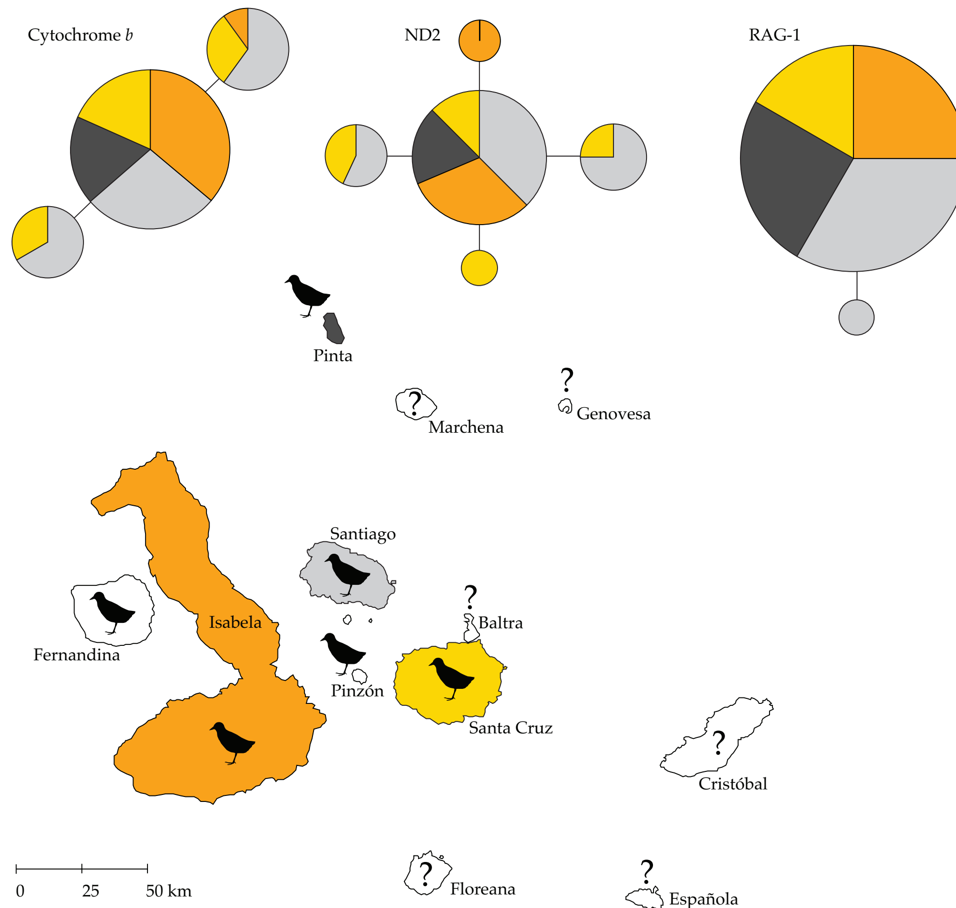

307
308 **Figure 2.** Haplotype network of mtDNA cyt*b*, ND2 and nuclear RAG-1 of *Laterallus spilonota* over
309 a map of the Galápagos Islands. Each circle represents a different haplotype, the size of the circles is
310 proportional to the number of individuals sharing that particular haplotype, and the color
311 corresponds to the four islands analyzed here. Haplotypes differ from each other by one nucleotide
312 represented by one branch. Islands with black rails represent the current distribution and question
313 marks correspond to islands where rails are now extinct, or their presence is unknown.

314 Our phylogenetic reconstruction places the Galápagos Rail as sister to the Black Rail, confirming the
315 suspicions by Leck [62]. Our findings also confirm the taxonomic placement for both *Laterallus* species,
316 together with the Inaccessible Island Rail and Dot-winged Crake, within a clade defined as the "American
317 black rails" with striking similarities in plumage coloration [31,47,63]. Additionally, and following island
318 biogeographic theory [64], we suspected sister relationships of species to be closer if their distributions are
319 also geographically close. In the case of the Galápagos avifauna, there are several examples of sister
320 relationships between land bird species on the islands to continental species (Central America: Galápagos
321 Yellow Warbler *Setophaga petechia aureola* [61], North America: Galápagos Hawk *Buteo galapagoensis*
322 [65], North/Central America: Galápagos Flycatcher [60], South/North America: *Pyrocephalus* flycatchers
323 [59]) and Caribbean forms (Darwin's finches [66], Galápagos mockingbirds genus: *Mimus* [10]). The
324 Black Rail presents a patchy distribution with resident subspecies populations on both the east (*L. j.*
325 *jamaicensis*) and west (*L. j. coturniculus*) coast of North America (mainly US), Caribbean and Central
326 America, with non-breeding (migrant) populations wintering in the Gulf of Mexico and Caribbean (*L. j.*
327 *jamaicensis*) [67]. Another group of subspecies is distributed along the Pacific coast of Perú (*L. j.*
328 *murivagans*) and Chile (*L. j. salinasi*), and from high elevation marshes and lakes around Lago Junín (*L. j.*
329 *tuerosi*) at 4,200 m elevation in the central Andes of Perú [68,69]. It is likely that this species contains several
330 phylogenetically distinct groups given the large, geographically-disjunct distribution and defined migratory
331 behavior, and *L. j. tuerosi* has indeed been suggested to represent a distinct species [63,70]. Leck's suggestion

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332 for the origin of the Galápagos Rail as derived from former migrants of North American Black Rail
333 populations (*L. j. jamaicensis*) needs further exploration by including phylogenetic affinities between all
334 Black Rail populations to pinpoint a possible origin of the Galápagos Rail. If proven right, it would not be
335 the first group of migratory birds to have reached the Galápagos Islands, followed by the loss of this
336 behavior to become a resident (and endemic) species of the archipelago (e.g. *Pyrocephalus* flycatchers,
337 Galápagos Yellow Warbler).
338 The little genetic differentiation recovered from rail populations between islands is nevertheless
339 surprising, particularly given the natural history of the species and time since colonization. Most insular
340 rails are endemic to one island (or set of islands), suggesting a limited vagility after arrival [47,71,72].
341 Contrary to these notions, our haplotype reconstruction suggests high degrees of connectivity between
342 islands. The absence of genetic structure in the three markers could be attributed to frequent movements
343 among the islands that are on average 25 km apart, with the largest distance to Pinta 75–90 km from
344 neighboring islands containing rail populations. These results were contrary to our prediction that
345 Galápagos Rails should show higher levels of genetic structure not only based on its limited dispersal ability,
346 but also given its habitat specialization. Rails are restricted to patchy marshes and meadows in the highlands
347 and thus potentially support smaller local population sizes. It is important to mention that in the past, rails
348 have been seen foraging near the coast in mangrove habitat [31], possibly increasing the chances to access
349 to open water. It is possible then, that rails perform seasonal (or random) elevational migrations towards
350 the coast and thus increase the chance for between-island connectivity as shown by the genetic sharing of
351 haplotypes. A degree of swimming capacity reported for this species could explain this pattern [73], but
352 alternative means of dispersal (i.e. rafting, nocturnal flights) are mere speculations at this moment.
353 Low levels of genetic diversity reported in Galápagos Rails could be indicative of the negative effects
354 of past population bottlenecks. Galápagos Rails have suffered dramatically from the introduction of
355 invasive species, either directly or indirectly. Goats were introduced after the first human settlements on
356 the islands, with the largest impact in the 1960s and 1970s. Grazing goats stripped bare large expanses of
357 native highland habitat, crucial for the presence of Galápagos Rails and other endemic fauna (giant
358 tortoises). This ecological erosion combined with the presence of rats and cats, probably extirpated
359 populations on Floreana, Baltra, Cristóbal, and Pinta islands [31,73,74] and most likely dramatically
360 reduced populations numbers on surviving islands, as our homogenous genetic data indicates. After
361 aggressive goat eradication programs and pest control programs were put in place, many of these islands
362 became goat free (currently Isabela, Pinta, and Santiago). But not all islands suffered the impact of mammal
363 introduction at the same magnitude. Varying levels of genetic diversity found across islands could be the
364 result of such idiosyncratic historical events shaping rail demography on each island separately. For
365 instance, Pinta populations showed the lowest diversity compared to other islands (mtDNA markers).
366 Pinta was reported to be practically rail free after the introduction of goats [75], with a swift recovery only
367 a few years after the start of eradication programs in late 1971 [73]. Currently rails are commonly found in
368 high numbers thriving in the now recovered habitat. These past events could have left a genetic signature
369 in present-day individuals, descendants from a reduced genetic pool of surviving birds. Alternatively, a
370 complete extirpation on Pinta followed by a recolonization event from neighboring islands (founder effect)
371 could also result in this pattern. The lack of private haplotypes on Pinta and the sharing of common
372 haplotypes across islands supports the notion of high connectivity following island bottlenecks (or local
373 extinction) and possible dynamic across-island recolonization events.
374 Natural history collections have served as a source of invaluable material to explore changes in
375 declining, extinct or inaccessible taxa. Here, we relied on historical samples collected by the California
376 Academy of Sciences in 1905–1906 to evaluate, for the first time, the phylogenetic reconstruction of the
377 endemic Galápagos Rail. The combination of historical and modern samples allowed us to put forward a
378 glimpse of the genetic history for this species and explore critical aspects of its evolutionary history.
379 Unfortunately, several islands lost their rail populations in the last few decades (Floreana, Cristóbal, Baltra)
380 and the magnitude of the historical loss of genetic variation could be great. Museum specimens collected

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381 prior to this ecological collapse (including from islands on which they are now extinct) could be used to
382 inform the magnitude of the effect of invasive species on endemic Galápagos species. Genetic data
383 produced from both present and historical samples has the potential to guide *in situ* management as well as
384 translocations and reintroductions to islands currently with extirpated populations. The overall
385 vulnerability of Galápagos Rail populations on the Galápagos is mirrored by the precarious conservation
386 status of most insular rails around the world. Because the Galápagos Rail is inconspicuous, and one of the
387 least studied land bird species on the Galápagos, its decline and extinction could go unnoticed. The
388 implementation of our suggested framework in conjunction with the Galápagos National Park
389 recovery/reintroduction program could directly benefit this endemic species and change the historical
390 extinction record of flightless rails.

391 **Supplementary Information:** The following are available as supplementary Information (pdf): Figure S1:
392 Graphic representation of mitochondrial genome assembly, Table S1: Primer information, Table S2:
393 Sequencing characteristics of mitochondrial genome, Table S3: Mitogenome sequence variation among
394 museum samples, Table S4: Sequence information for mitochondrial cyt*b* and ND2, and nuclear RAG-1
395 markers.

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398 and M.S.; formal analysis, JA.C., P.J.M.T., S.E.U., A.C.B., and M.S.; investigation, J.A.C., P.J.M.T., S.E.U.,
399 J.G.L., A.C.B., E.A.D. and M.S.; resources, J.A.C., S.E.U., A.C.B., and M.S.; data curation, J.A.C., A.C.B., and
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416 Data Accessibility

417 The new Galápagos Rail sequences (cyt*b*, ND2, RAG-1 alleles) have been deposited at GenBank with the
418 accession numbers MW074873–MW074882 and mitochondrial reference genome with the accession
419 number MW067132. Input and output files from the phylogenetic analyses have deposited at Zenodo:
420 https://doi.org/10.5281/zenodo.4046620.

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