Evolution, 2026, **0**(**0**), 1–14
https://doi.org/10.1093/evolut/qpag068
Advance access publication 6 May 2026
Original Article

Founder effects drive high mitochondrial dN/dS in island rails

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Abstract

Insular (island-limited) populations typically show signatures of weak purifying selection, indicating high genetic load and reduced fitness compared with mainland populations. However, the source of this pattern is often unclear—it may reflect residual signatures from a temporary period of small effective population size (Ne) associated with island colonization (founder effects), persistently small Ne due to the lower carrying capacity of islands (range limitations), or relaxed selective constraints unrelated to Ne. Here, we disentangle these hypotheses by analyzing the drivers of variation in evolutionary rates of nonsynonymous (dN) and synonymous (dS) sites in nine mitochondrial genes (8001 bp) from 40 rail species (Aves: Rallidae). We find that insular species with short terminal branches (indicating recent island colonization) have highly elevated mitochondrial dN/dS across multiple mitochondrial genes. In contrast, rails representing more ancient island colonizations have dN/dS ratios that are indistinguishable from mainland/widespread species. Furthermore, we find that island size is unrelated to dN/dS among island species. These results indicate that insular rails suffer a high initial cost of island colonization and undergo a period of inefficient selection due to founder effects, but that there is little impact from longer-term range limitations or relaxed selection.

Keywords: population genetics, founder effects, purifying selection, island biogeography, dN/dS, Rallidae

Introduction

Island populations typically exhibit signatures of low effective population size (Ne), namely inefficient purifying selection and low genetic diversity when compared with mainland relatives. This pattern has been demonstrated in a vast number of avian systems—including ducks and doves (Johnson & Seger, 2001), corvids (Kutschera et al., 2020), tits (Stervander et al., 2015), mockingbirds (Vlček et al., 2025), song sparrows (Wilson et al., 2009), tanagers (Brüniche-Olsen et al., 2019), and in studies comparing multiple avian groups (James et al., 2016a; Leroy et al., 2021; Recuerda et al., 2024); although see Wright et al. (2009) for an exception to this trend. More widely, the pattern of reduced insular effective population size appears consistent across terrestrial vertebrates (Frankham, 1996; James et al., 2016a; Woolfit & Bromham, 2005), while the evidence in plants and invertebrates is more mixed (García-Verdugo et al., 2015; Hamabata et al., 2019; James et al., 2016a; Levin & Miller, 2021). Determining the drivers of effective population size variation is a key aim in conservation biology, given that effective population size impacts both the adaptive potential of a population and its capacity to purge harmful mutations (Charlesworth, 2009; Hoffmann et al., 2017). However, the primary process driving low effective population sizes on islands remains unclear, as there are two distinct stages to island colonization that are predicted to cause a reduction in Ne.

First, the initial founding of an island population by a small number of individuals is expected to result in a population bottleneck and a period of very small population size (Nei et al., 1975). Second, after the founding population expands to fill the island and its effective population size re-

Received October 8, 2025; revisions received April 2, 2026; accepted April 7, 2026
Associate Editor: Yvonne Willi; Handling Editor: Luis-Miguel Chevin

covers from the initial colonization event, the geographical constraints of the island may impose a strict limit to the population's carrying capacity (Frankham, 1996). These explanations represent distinct processes and will here be termed the "founder effects" and "range limitations" hypotheses, respectively. Understanding the relative importance of these different processes has clear conservation implications. If historic founder effects drive signatures of low Ne, then the efficiency of purifying selection is expected to increase once the population recovers from its initial bottleneck, halting further increases in genetic load and eventually reversing the damage through positive selection on compensatory mutations (or back mutations). If range limitations drive signatures of low insular Ne, established populations on small islands will continue to build up genetic load, leading to more permanent reductions in fitness (Bertorelle et al., 2022; Lohr & Haag, 2015; Willi et al., 2013). In extreme cases, the continued build-up of genetic load may lead to mutational meltdown and extinction (Lande, 1994; Lynch & Gabriel, 1990).

Previous comparative research has provided support for both the range limitations and founder effects hypotheses. The range limitations hypothesis predicts that smaller islands will have smaller effective population sizes than larger islands, both because the maximum carrying capacity is reduced, and because more severe population fluctuations are expected in smaller populations (Frankham, 1996; MacArthur & Wilson, 2001). This prediction is well supported by studies of island size and genetic diversity, which have often indicated that populations occupying smaller (or fewer) islands are less genetically diverse [e.g., in plants (García-Verdugo et al., 2015; Juan et al., 2004), reptiles (Harradine et al., 2015), amphibians (Wang et al., 2014), and mammals (White & Searle, 2007)]. There is particularly strong evidence for reduced genetic diversity on smaller islands for passerine birds (Brüniche-Olsen et al., 2019; Frankham, 1996; Gyllenhaal et al., 2025; Vlček et al., 2025), although comparative studies across wider avian groups have not recovered the pattern (James et al., 2016a; Woolfit & Bromham, 2005). In birds, the range-limitations hypothesis is further supported by studies looking more widely across species (not just island-limited groups), which have shown that species with larger geographic ranges exhibit greater genetic diversity and signatures of more efficient purifying selection (Brüniche-Olsen et al., 2021; Clark et al., 2023; Wanders et al., 2023).

The founder effects hypothesis predicts that populations representing more recent colonizations should exhibit stronger signatures of low Ne, while populations representing more ancient colonizations are expected to have recovered following the initial costs of colonization. Consistent with this expectation, lower genetic diversity has been detected in more recently colonizing populations in plants (García-Verdugo et al., 2015; Veltjen et al., 2023), as well as in a comparison of multiple species groups, including invertebrates and birds (James et al., 2016a). However, the opposite pattern is seen when comparing genetic diversity among white-eye (Zosterops) populations (Clegg et al., 2002; Sendell-Price et al., 2021). Studies of sequential colonization events in passerine birds provide particularly strong evidence for founder effects reducing insular Ne, as populations that have undergone multiple colonization events exhibit lower genetic diversity than populations that have undergone a single colonization event (Clegg et al., 2002; Gonzalez-Quevedo et al., 2015; Martin et al., 2023; Nelson-Flower et al., 2018; Sendell-Price et al., 2021; Spurgin et al., 2014; Wilson et al., 2009). Finally, the observation that mainland bird populations exhibit signatures of reduced Ne when they have been founded from island populations provides further evidence that colonization itself can lead to reduced Ne even in the absence of range limitations (Charlesworth & Eyre-Walker, 2007). Ultimately, both founder effects and range limitations are likely to cause reductions in insular effective population size, with consequences for selection efficiency. However, further research is required to identify the life history traits and geographical contexts underlying variation in the impact of each process. For example, more severe founder effects may be expected when an island is colonized by fewer individuals, and for species where population expansion occurs slowly (Nei et al., 1975). On the other hand, more severe range limitation effects are expected for smaller islands and for species with low population densities (Frankham, 1996).

Rails (Aves: Rallidae) have long been noted for their propensity to colonize remote islands (Olson, 1973), and may once have been represented by as many as 460–1,600 flightless island-endemic species in the Pacific alone (Steadman, 2006). There is pronounced variation among island colonization events in this group, with colonized islands varying in size from tiny isolated coral atolls (such as Laysan Island) to far larger continental-like islands (e.g., New Zealand's North and South islands), and with colonizations occurring both in ancient (>10 mya) and very recent (<125 kya) times (Garcia-R et al., 2014; Slikas et al., 2002). Phylogenetic data indicate that most island species originate from high Ne ancestral populations with broad distributions, rather than following a stepping-stone colonization pattern from neighboring islands (Diamond, 1974; Kirchman, 2009, 2012; Slikas et al., 2002; Trewick, 1997). This pattern likely reflects the tendency of rails to rapidly evolve flightlessness once released from predation pressure, which precludes further colonization (Steadman, 2006). Consistent with flightlessness being key to this pattern, one known exception to the rule of independent colonization events is the stepping stone colonization of New Zealand's South Island from North Island (or vice versa) by an ancestral takahē, which is thought to have occurred over land prior to the formation of the Cook Strait (Verry et al., 2024). The tendency toward flightlessness and the rarity of stepping stone colonization make insular rails a particularly interesting case study for understanding the drivers of low effective population size on islands, as each island species can typically be viewed as the result of a unique founding event (Kirchman, 2009). However, the widespread extinction of insular rails and the associated difficulty of gathering genetic data have so far prevented any between-species comparative genetic study in this clade.

The mitochondrial dN/dS ratio (the ratio of non-synonymous substitutions to synonymous substitutions) and the related Kr/Kc ratio (the ratio of radical non-synonymous substitutions to conservative non-synonymous substitutions) provide attainable measures of selection efficiency for clades with little genetic data available, as they can be calculated from a single mitochondrial genome per species. DN/dS is most appropriate for analyzing short branches where mutation saturation is not an issue (as in the current study), while the Kr/Kc ratio is more appropriate

for analyzing very long branches where synonymous substitutions can become saturated (Nabholz et al., 2013). Since the majority of vertebrate mitochondrial non-synonymous substitutions are fixed through genetic drift rather than positive selection (James et al., 2016b), and since genetic drift is stronger in small populations, there is a strong theoretical expectation for these ratio statistics to correlate negatively with effective population size (Ohta, 1992). This expectation has been borne out in comparative studies of mammals and birds, which have demonstrated that species with larger effective population sizes exhibit lower dN/dS or Kr/Kc ratios (Nabholz et al., 2013; Popadin et al., 2012; Woolfit & Bromham, 2005).

Here, we generate a dataset comprising mitochondrial protein-coding sequences from 58 rail species, including 29 newly sequenced species, to investigate the drivers of weak purifying selection in island rail populations. We calculate terminal branch dN/dS from the nine best-represented mitochondrial genes as a signature of selection efficiency since island colonization. We first show that insular rail populations do indeed show elevated mitochondrial dN/dS ratios, before investigating the variation in dN/dS within insular species to disentangle the competing explanations for weak selection efficacy. We test whether dN/dS is particularly elevated for more recent island colonizations, as predicted by the founder effects hypothesis. We test whether dN/dS ratios are particularly elevated on smaller islands, as predicted by the range limitations hypothesis. Finally, we test whether elevated dN/dS ratios are restricted to a subset of mitochondrial genes or sites, which would indicate a role of positive selection.

Methods

Assembly, annotation, and alignment of mitochondrial genomes

We collated all available mitochondrial gene sequences from 78 gruiform species for this study, including 48 mitogenomes collated from published data, and 30 mitogenomes generated from new sequencing data (29 new rail mitogenomes and 1 new crane mitogenome). We extracted protein-coding mitochondrial genes for an initial set of 48 species using Mitofinder 1.4, using published mitogenome assemblies for 43 species and sequencing data for five species (using the reference mitogenome for *Zapornia akool*, accession no. NC_023982, as a seed sequence) (Allio et al., 2020). We aligned these genes using MAFFT (v7.294b) (Katoh & Standley, 2013, as in Berv et al., 2024). We then supplemented this preliminary dataset with additional mitochondrial sequences from 30 species, two of which were recently published and the remainder generated from new sequence data following one of two workflows:

(1) For 16 extant species, mitogenomes were derived from single-tube long fragment reads generated for upcoming B10k genomes (Wang et al., 2019; Zhang, 2015), using the default settings of NOVOPlasty (v2.7.2) (Dierckxsens et al., 2017), an initial *K*-mer size of 39, and the Red Junglefowl published mitogenome (NC_040902) as a seed sequence. If assembly failed, smaller *K*-mer sizes (23 or 19) were applied and larger memory allocations were tested to obtain the best assembly. If multiple assemblies were generated, the circularized sequence with the fewest ambiguous bases was chosen, or if no circularized mitogenome was produced, the longest contig was chosen.

(2) For seven extinct species and a further five extant species, museum toepad samples were sequenced using 150 bp paired-end Illumina reads, which were quality-trimmed and merged into single-end data using fastp (Chen et al., 2018) due to the presence of short insert sizes and many overlapping paired reads. The workflow for DNA extraction and library preparation of these toepad samples is described in detail by Irestedt et al. (2022). Mitogenomes for these species were generated by applying the default settings of GetOrganelle (v1.7.7) as NOVOPlasty does not support single-end data, and the published mitogenome of *Z. atra* was used as a seed sequence (MN356311) (Jin et al., 2020). Assembly graphs for these assemblies were then visualized and disentangled manually using Bandage (v8.1) (Wick et al., 2015).

We annotated all 28 newly assembled mitogenomes, and the two recently published mitogenomes, using MitoAnnotator (v4.05) (Iwasaki et al., 2013), and added the protein-coding regions to the existing gene alignments using MUSCLE algorithm implemented in MEGA (v11) (Edgar, 2004; Tamura et al., 2021). We checked and adjusted all alignments manually and removed species if >10% of sites were missing for a given gene. To assess methodological biases, we generated mitochondrial protein-coding sequences for *Gallirallus philippensis* using all three assembly methods. Differences between assembly workflows were limited to trailing bases at the start and end portions of genes, which we trimmed from the alignments. Overlapping portions of genes were also trimmed away. This procedure resulted in alignments of all 13 mitochondrial protein-coding genes for all 78 species (although some species had missing data).

Cytosine deamination leads to increased C→T error rates in DNA libraries derived from degraded samples. However, since errors for any particular site are present in only a small minority of reads (1%–2% in highly degraded samples, Rathbun et al., 2017), they are expected to be trimmed away by GetOrganelle during the assembly of a consensus sequence (Jin et al., 2020). To confirm that C→T errors were indeed being excluded from our assemblies derived from museum skins, we compared the GC content of mitochondrial protein sequences, and found no evidence of a reduced GC ratio for historic DNA samples (Supplementary Figure 1).

Phylogeny

To construct a phylogenetic tree, we concatenated all 13 mitochondrial protein-coding genes into a single alignment and used the ModelFinder software implemented by iqtree (v2.2) (Kalyaanamoorthy et al., 2017; Minh et al., 2020), which identified an MGK+ F3 × 4 + R7 codon substitution model as the most appropriate (minimizing the Bayesian information criterion). This model includes separate synonymous and non-synonymous substitution rates and allows unequal nucleotide frequencies across the three codon positions. We assessed support using 1,000 ultrafast bootstrap replicates to assess tree consistency (Hoang et al., 2018), and almost all

nodes had high bootstrap support (see Supplementary Figure 2 for the complete tree).

Initial data filtering

To ensure accurate estimation of dN/dS estimates across the phylogeny, we retained only species whose terminal branch was placed with >85% support (with five species being removed due to unclear placement). Note that the deep nodes representing the divergence of the major Rallidae tribes show low bootstrap support and a different order of splitting than previous studies (Garcia-R et al., 2020; Gaspar et al., 2020; Kirchman et al., 2021). However, given that few substitutions occurred between these deep divergence events, this issue will have little impact on the dN/dS estimates of terminal branches. Other than these deep nodes, our mitogenome-based tree topology is highly concordant with the previous phylogenies (Garcia-R et al., 2020; Gaspar et al., 2020; Kirchman et al., 2021).

To maximize the number of species in the study and avoid biases associated with missing data, we only analyzed the nine best-represented protein-coding genes (ATP6, COX1, COX2, COX3, CYB, ND1, ND4, ND4L, and ND6), and we removed four species with missing data from these nine genes. We removed two further species because their terminal branches were too short for accurate dN/dS estimation. These initial filtering steps produced an alignment of nine genes from 67 gruiform species, with <10% missing data per species per gene. See Supplementary Table 1 for details of the species filtered at each step. We used the phylogeny produced using all 78 species for all analyses, with the excluded species pruned. ALTER was used for file format conversions (Glez-Peña et al., 2010).

Free-ratios model

We concatenated all nine of the well-represented mitochondrial genes into a single alignment (8001 bp), and calculated dN, dS, and dN/dS for the terminal branches of all 67 species that passed the initial filtering steps using the free-ratios model implemented in PAML (v 4.9) (Yang, 2007). The free-ratios model uses a codon alignment to estimate dN and dS separately for each branch in a phylogenetic tree with a maximum likelihood approach. We also ran the free-ratios model separately for each gene, to test for mutation saturation, and excluded six species with a terminal branch $dS > 1$ for any of the nine genes. To assess the reliability of our dN/dS estimates, we produced 100 bootstrap replicates from our 8001 bp alignment by resampling codons, and reran the free-ratios model for each of these alignments. The bootstrap replicates showed that dN/dS estimates are increasingly unreliable when based on fewer substitutions (Supplementary Figure 3). On the basis of these results, we excluded species with fewer than 70 terminal-branch substitutions to avoid the least accurate dN/dS estimates (four species). Note that retaining species with 50–70 substitutions (*Laterallus spilonota*, *Atlantisia rogersi*), or excluding species with 70–100 substitutions (*Z. atra*, *L. jamaicensis*, and *L. spiloptera*), led to very similar results. However, also retaining the very shortest branches (~40 substitutions, *Z. palmeri*, *Z. pusilla*) led to notably weaker patterns, presumably due to the instability of dN/dS estimates based on few substitutions.

We removed non-rallid species from our main analyses to reduce variance, since all the insular species passing filtering checks were in the Rallidae family, and because other Gruiformes have notably different life history traits (e.g., far larger body mass in the cranes). The dataset of species-specific dN/dS ratios thus consisted of 40 rails (11 insular and 29 non-insular); see Figure 1.

Life history data

We considered a species to be "insular," if it was restricted to an island or archipelago with an area <300,000 km^2—this broad definition avoids excluding the weka *G. australis*, which exhibits a typical flightless "island" phenotype, despite occupying both islands of New Zealand (combined island size ~257,000 km^2). Note that rerunning analyses with a lower limit of 250,000 km^2 (as in Sandvig et al., 2019), or with no limit at all (including Madagascar and New Guinea as islands), produced very similar results and no change to model interpretations. Note that only two of the insular species in this study are able to fly (the Galapagos crake *L. spilonota* and the Hawaiian coot *Fulica alai*), and neither of these species passed the QC steps necessary to produce accurate species-specific dN/dS estimates, so the impacts of flightlessness and insularity cannot be disentangled. We used estimates of island size primarily from Dahl (1991), supplemented by Bryan (1959) and Broad and Oliveros (2004). To test whether insular species actually occupied smaller ranges, we extracted estimates of species-specific geographic range sizes, including estimated historic ranges for extinct species, from BirdLife International (BirdLife International & Handbook of the Birds of the World, 2023) shapefiles, and assessed the impact of insularity on geographic range size with a phylogenetic *t*-test (using the "phylANOVA" command of the phytools package, Revell, 2012). Species names follow the AviList v2025 taxonomy (AviList Core Team, 2025).

We also collated data on possible confounds to our analyses—tarsus length as a proxy of body mass (Rising & Somers, 1989), due to the association between body mass and the effective population size (Nabholz et al., 2013; Wanders et al., 2023); the time since island settlement by humans, given the well-documented destructive impact of human settlement on island rail populations (Steadman, 2006); and the equilibrium GC content of fourfold degenerate sites (GC4*), due to the association between mutation biases and dN/dS ratios (Nabholz et al., 2013). We collated tarsus length data from the Avonet database for extant birds (Tobias et al., 2022), and from the Avotrex database for extinct birds (Sayol et al., 2024). Tarsus length was used rather than body mass because it can be directly measured from the skins and skeletons of extinct species, rather than relying on imputation (body mass data were unavailable for 4/11 of our insular species with species-specific dN/dS estimates). Furthermore, preliminary analyses found that tarsus length and body mass showed a strong log-linear correlation among the insular rails included in this study ($R^2 = 0.93$, data included in Supplementary Table 1). We took estimates of the number of years since island settlement by humans from Lévêque et al. (2024), these estimates were available for all island rails except *G. calayanensis* and *G. insignis*. The Inaccessible Island rail *L. rogersi* was also excluded from models including human settlement, given that Inaccessible Island remains

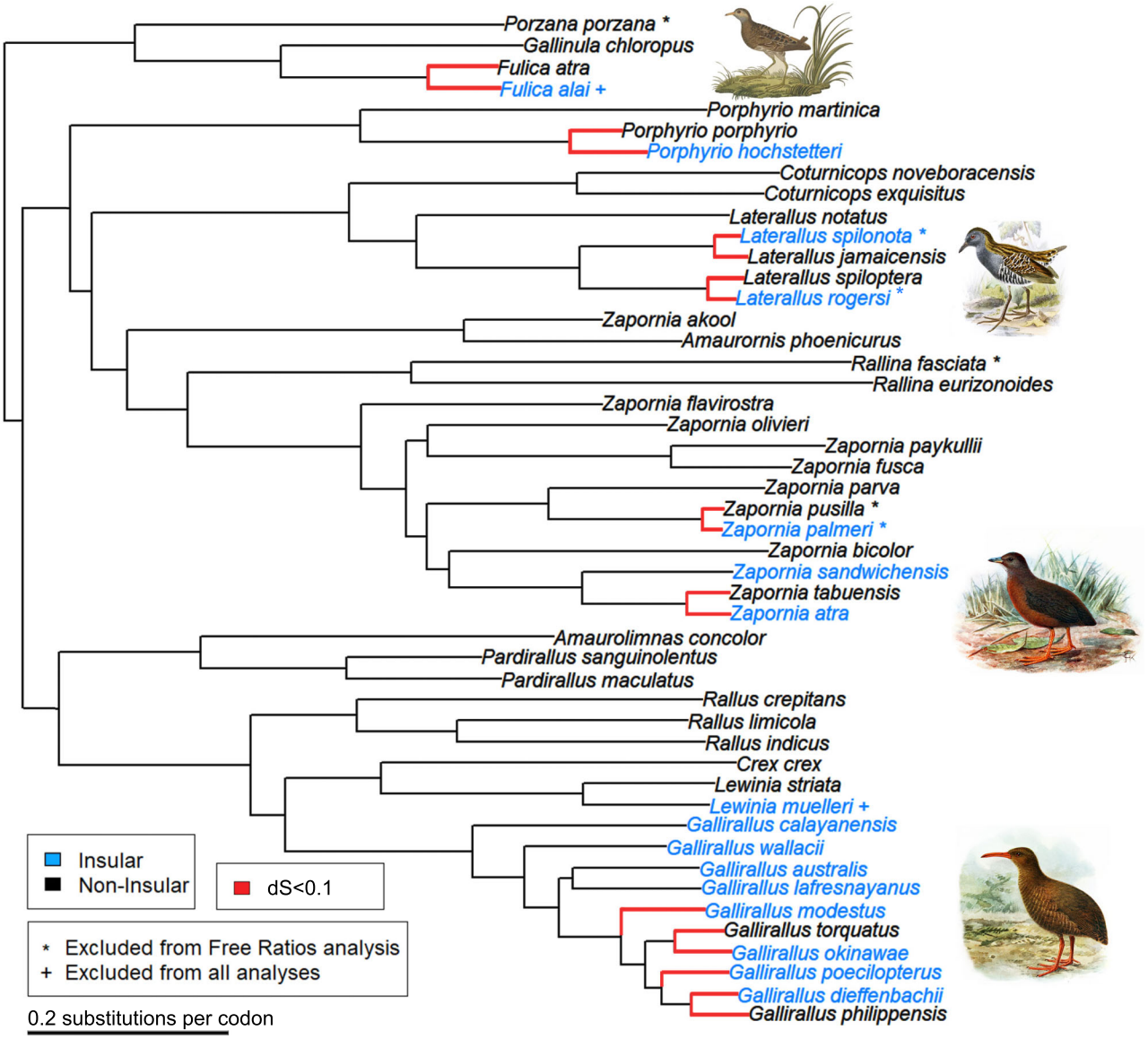

Figure 1. Rallidae phylogeny showing all species included in the analyses of dN/dS, with insular species indicated in blue. Species with evidence of saturated mitochondrial evolution (dS > 1) or too few substitutions (<70 in total) for branch-specific dN/dS estimates are indicated with a "∗" and we excluded these from the "free ratios" analysis, which requires accurate dN/dS ratios for every branch. We retained these species for the two-ratio and three-ratio models as well as the BUSTED and RELAX models. "Short" terminal branches (dS < 0.1) are shown in red to highlight the species where insular dN/dS is elevated according to the two-ratio vs. three-ratio analysis. *Lewinia muelleri* and *Fulica alai* are also included in the tree, despite being excluded from all analyses, to accurately show the terminal branch length leading to *Lewinia striata* and *Fulica atra*. Illustrations are adapted from public domain images (Durnford, 1877; Nozeman & Houttuyn, 1770; Rothschild, 1907).

unsettled. GC4∗ was calculated using CoEvol (v1.6), with a model that included only fourfold degenerate sites (Lartillot & Poujol, 2011).

We estimated the branch lengths variable (dS) as the number of synonymous substitutions accumulated per site in the terminal branch (the branch in the phylogeny immediately leading to the sampled species), using estimates from the free-ratios model implemented in PAML (Yang, 2007). We calculated this parameter to approximate the number of generations since island colonization: species that have only recently colonized islands are expected to have been isolated from their non-insular sister species for fewer generations, and so will have accumulated fewer neutral substitutions [there is little evidence for selection on synonymous sites in animal mitogenomes (Jia & Higgs, 2008)]. Our approach resembles James et al. (2016a), and it assumes that island colonization is closely associated with the divergence of a given insular species from its non-insular sister species. For almost all insular species in our dataset, this was a plausible assumption as the dataset includes sequencing data from the closest non-insular relative. However, the sister taxa of two insular species [*Lewinia muelleri* and *F. alai*, based on Garcia-R et al., (2020) and Gaspar et al. (2020)] remain unsampled, so the number of synonymous substitutions in the terminal branch is expected to over-estimate the number of generations since island colonization. We therefore removed *F. alai* and *L. muelleri* from our statistical analyses. Note that other insular species may have similar biases, if their ances-

tral source populations split from the extant non-insular lineage without becoming insular, then became extinct without leaving a related non-insular descendant population. However, assuming that island colonization is the more likely cause of branch splits leading to insular species, the accumulation of synonymous substitutions along terminal branches will reflect the number of generations since island colonization.

Statistical analysis of species-specific dN/dS

Our statistical analysis included three main models: a phylogenetic *t*-test to establish whether insular species exhibited higher dN/dS ratios than non-insular species, and two PGLS (phylogenetic generalized least squares) models. The first PGLS model tested whether insular dN/dS ratios were particularly high for species with low terminal branch lengths (indicating recent island colonization), and the second PGLS model sought tested whether insular dN/dS ratios were particularly high for species on smaller islands. The two PGLS models could not be combined into a single analysis because analysis of branch lengths necessitated the inclusion of non-insular species, while analysis of island size necessitated the exclusion of non-insular species (discussed below). We note that phylogenetic control may not strictly be necessary for these analyses, since terminal-branch dN/dS and rail insularity are traits associated with evolution along the terminal branch only. However, the impact of confounds with strong phylogenetic signal (e.g., body mass) can also be excluded through the use of phylogenetically controlled analyses.

To avoid over-fitting our linear models by including all possible terms, we tested whether our potential confounding variables (tarsus length, years since human settlement, and GC4∗) were correlated with our explanatory variables (branch length and island size). We also tested whether branch length correlated with island size, to ensure our hypotheses were independent, and used a *t*-test to check whether branch length correlated with insularity. Note, we do not control for phylogeny in these tests, because they are intended to discover confounds in our analysis, rather than test for genuine relationships among life history traits. These test models detected a potentially confounding relationship between island size and tarsus length, with larger birds being located on larger islands (Supplementary Table 2). Given the central role of human activity in the extinction of insular rails (Lévêque et al., 2024; Steadman, 2006), this trend seems likely to reflect selective extinction of smaller species, rather than a biological tendency for larger species to be present on larger islands (Lévêque et al., 2024). This potential confound was resolved by including tarsus length as a covariate our PGLS models (details below), and by checking the variance inflation factors of these models using the "vif" command of the car package in R (Fox et al., 2012). Test models also revealed a potentially confounding relationship between insularity and branch length, with non-insular species having longer branches than insular species. Visual inspection of the data revealed that the relationship was driven by the presence of several very long branches leading to non-insular species, and the correlation could be removed by excluding species with a branch length >0.21 (Supplementary Figure 3, Supplementary Table 3). The final dataset for analysis of the species-specific dN/dS ratios therefore included 28 rails (11 insular and 17 non-insular).

DN/dS ratios can be elevated when branches are short even in the absence of founder effects, due to the impact of sampling variance or due to the influence of unfixed polymorphisms (Wolf et al., 2009). To control for these impacts, we retained non-insular species in our PGLS model investigating the impact of branch length, which tested for an interaction between dS and insularity affecting dN/dS. In this context, the founder effects hypothesis predicts a steeper relationship between dS and dN/dS in insular species than in the non-insular control group. We note that in extremely large populations where genetic drift is essentially absent, there is an expectation for dN/dS to show a non-linear relationship with dS, and for dS/dN to show a more linear relationship (Rocha et al., 2006). However, in our dataset of rails with limited population sizes, dN/dS showed a stronger linear relationship with dS than dS/dN, and dS/dN was found to be highly heteroskedastic (with variance scaling with dS), making it inappropriate for linear model analysis. We therefore opted to use dN/dS as a more appropriate dependent variable for this model.

Our second PGLS model tested whether island size (or geographic range size) affected dN/dS ratios, as predicted by the range limitations hypothesis. Non-insular species could not be retained in these analyses due to a very strong correlation between insularity and geographic range size (and because island size has little meaning for non-insular species). However, dS was retained as a covariate. We note that island topography is also important for the persistence of wild populations, as low-lying islands may undergo frequent bottlenecks or local extinctions due to cyclones and high water stands (e.g., Hume & Martill, 2019). However, preliminary analyses found that excluding the parts of a species' range with an elevation <10 m had little impact on the results (these data are included in Supplementary Table 1).

Two-ratio and three-ratio models

The free-ratios analysis approach is uniquely informative about how consistent patterns are across groups of species. However, our bootstrap analysis revealed high confidence intervals for terminal-branch-specific estimates of dN/dS generated by the free-ratios model, which is likely to reflect the high number of parameters being estimated from branches with relatively few non-synonymous substitutions (Yang, 2007). To ensure overparameterization was not biasing the results, we therefore constructed a less complex test of the interaction between insularity and branch length, by comparing much simpler models that allowed only two or three unique dN/dS ratios in the entire tree (as opposed to a unique dN/dS ratio for each branch). Since branch-specific dN/dS ratios were not required for these models, the rallid species that were excluded from the free-ratios analysis due to sequence saturation or the presence of too few substitutions could be retained. We also retained *L. muelleri* and *F. alai as* background branches for these models, since excluding them would alter the branch lengths of *F. atra* and *L. striata*. We again excluded non-rallid species. The two-ratio and three-ratio branch models were implemented in PAML using the same concatenation of all nine protein-coding genes that passed the initial filtering steps (8001 bp).

First, we constructed a null two-ratio model that allowed two different dN/dS ratios among branches, with one dN/dS ratio for terminal branches with dS < 0.1 (n = 17, high-

lighted in Figure 1) and one for all other (background) branches ($n = 31$). Next, we constructed a three-ratio test model that allowed three different dN/dS ratios among branches, with one dN/dS ratio for terminal branches with dS < 0.1 leading to insular species ($n = 9$) another ratio for terminal branches with dS < 0.1 leading to non-insular species ($n = 8$), and a third dN/dS ratio for all other (background) branches ($n = 31$). We then used a likelihood ratio test to evaluate whether the test model had a significantly higher log-likelihood than the null model, with a significant improvement indicating that dN/dS ratios vary significantly between insular and non-insular species when only short terminal branches are considered. Next, we constructed similar models based on longer branches (0.1 < dS < 0.21), a group that includes 10 non-insular species and 5 insular species. These models tested whether dN/dS ratios varied significantly between insular and non-insular species when only longer branches are considered. Similar to the free-ratios model analyses, the relatively low upper bound for dS (0.21) removed the potentially confounding association between insularity and dS (Supplementary Figure 3). As well as the main analysis that relied on a concatenation of all nine mitochondrial genes, the two-ratio and three-ratio models were also run separately for each gene to determine whether the elevated dN/dS seen in short-branch insular species was driven by a subset of mitochondrial genes.

BUSTED and RELAX models

To test whether the elevated dN/dS found in insular species with short branches reflected a small number of rapidly evolving sites under positive selection, or a more general relaxation/weakening of selection due to small effective population size or relaxed selection constraints, we implemented further models using the HyPhy software package (v2.5.36) (Kosakovsky Pond et al., 2020). These models used the same dataset as the two-ratio and three-ratio models. We used BUSTED to test for the presence of positive selection: insular species with short branches (dS < 0.1) were selected as the focal group ($n = 9$), and the model tested whether allowing site-specific dN/dS ratios > 1 for this group improved the model fit (Murrell et al., 2015). RELAX was then used to test for the presence of relaxed/weakened selection: insular species with short branches were again selected as the focal group ($n = 9$), while non-insular species with short branches were selected as a reference group ($n = 8$), and the model tested whether introducing a general increase (or decrease) in dN/dS ratios across all sites for the focal group improved the model fit (Wertheim et al., 2015).

NUMT identification

Nuclear mitochondrial DNA segments (NUMTs) are regions of nuclear genome that have been transposed from the mitochondrial genome (Hazkani-Covo et al., 2010). Following their introgression to the nuclear genome, NUMTs are no longer under selective constraint and can accumulate substitutions in sites that would be under strong purifying selection in the mitochondrion (Gherman et al., 2007). As a result, the accidental incorporation of NUMTs into mitogenome assemblies can lead to artificially high estimates of dN/dS and can bias comparative analyses (Lucas et al., 2022). This issue is expected to be particularly severe when NUMTs have been evolving in the absence of selection for significant periods of evolutionary time (i.e., ancient NUMTs).

To detect regions of our mitochondrial gene alignments that could be biased by ancient NUMT introgression, we separated the alignment into overlapping windows of 99 codons, with a step size of 33 codons. PAML free-ratio models were then applied to each window, and the rate of non-synonymous substitutions was extracted for each terminal branch (Yang, 2007). These models are highly parameter-rich, and since each is based on very little genetic data, we caution against any strong biological interpretation of the patterns in terms of insularity effects. Nevertheless, windows containing ancient NUMTs are expected to appear as window-specific and species-specific outliers in this analysis due to an elevated rate of non-synonymous substitutions. We found that no windows contained a species-specific dN > 0.05, and we visually inspected the substitutions occurring in all windows with a species-specific dN > 0.03 (the 12 greatest outliers, Supplementary Figure 4). For all such windows, the majority of substituted amino acids were not radically different from all other amino acids in that position of our alignment in terms of polarity or size (as defined by Weber et al., 2014). The substitutions in these regions therefore remained consistent with the presence of purifying selection, and did not indicate an influence of NUMTs.

In contrast to more ancient introgressions, recently introgressed NUMTs may have accumulated too few substitutions to be separated from true mitochondrial sequence using a scanning approach, but may still have a minor impact on dN/dS analysis. However, NUMTs are most likely to be incorporated into mitochondrial assemblies when the sequencing depth of nuclear DNA is similar to that of mitochondrial DNA. For all but one of our newly sequenced species, muscle tissue or toepad material was used for DNA extraction, both of which contain substantially higher copy numbers of mitochondrial DNA than nuclear DNA. NUMT inclusion is therefore unlikely for these species (Barker et al., 2015). However, one of the newly sequenced mitogenomes (*L. spiloptera)* was generated from a blood sample, where the risk of NUMT inclusion is much higher. To confirm that possible NUMTs in this assembly were not affecting our results, we repeated the dN/dS free-ratios analysis with *L. spiloptera* excluded.

Results

dN/dS is higher in recent island colonizers

A phylogenetic *t*-test revealed that dN/dS ratios estimated by the free-ratios model were significantly higher for branches leading to insular species (Figure 2A). Consistent with the founder effects hypothesis, a PGLS model revealed a significant interaction between dS (branch length) and insularity—dN/dS ratios were strongly negatively correlated with dS for insular species, while a far weaker relationship was detected in non-insular species (Figure 2B, Table 1). This result remained consistent when using the unrestricted dataset that includes a potentially confounding correlation between insularity and dS (Supplementary Figure 5A, Supplementary Table 4), when retaining cranes in the analysis (Supplementary Figure 5B, Supplementary Table 5), and when excluding the data from *L. spiloptera* (which

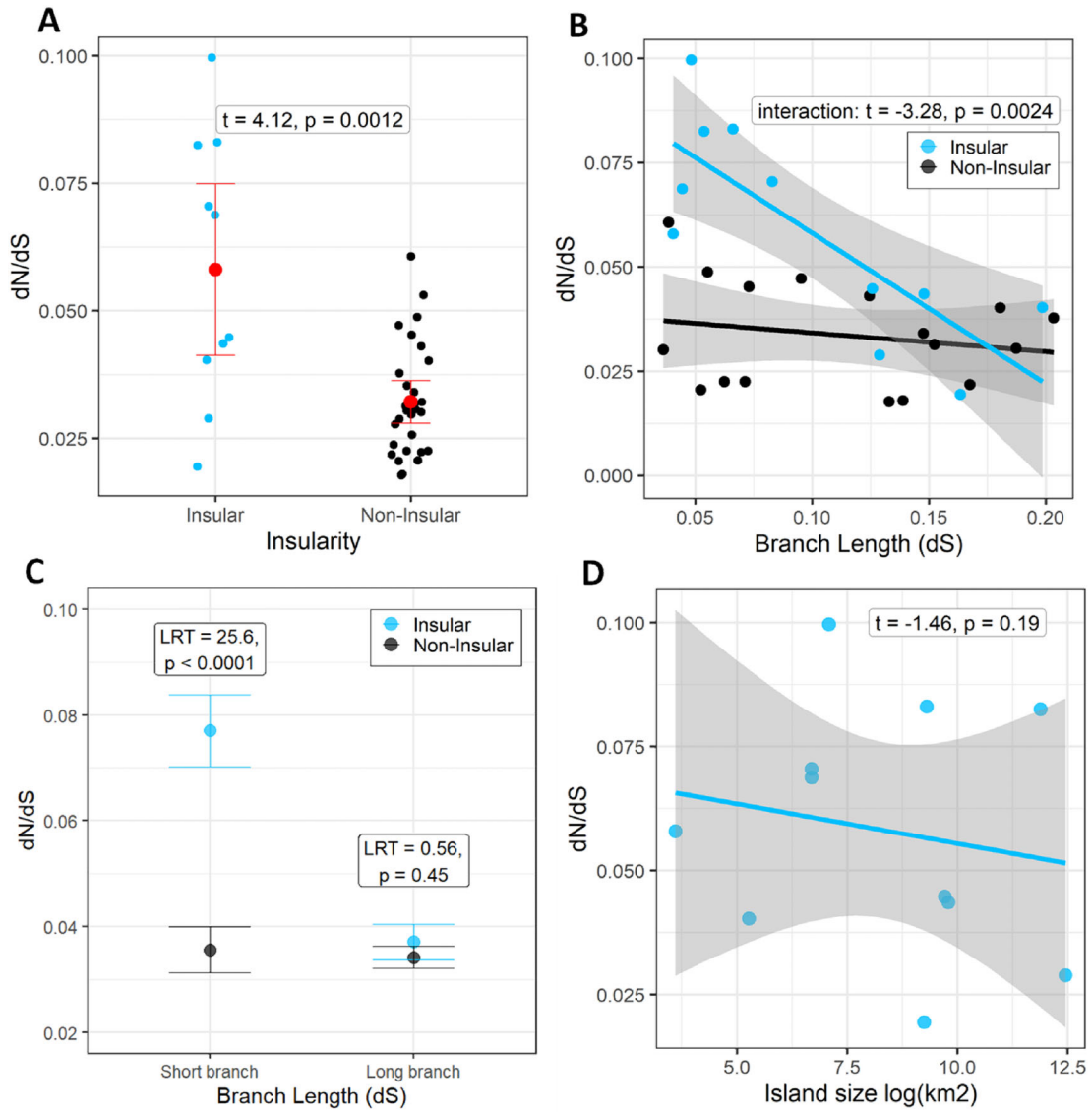

Figure 2. Evidence that insular species exhibit elevated dN/dS primarily due to a transient period of weak purifying selection. (A) Insularity is associated with significantly higher mitochondrial dN/dS. Data points reflect species, with means and 95% confidence intervals shown in red. Phylogenetic *t*-test results are reported. (B) Based on free-ratios models: insular species with shorter terminal branches show elevated dN/dS ratios, whereas no such relationship exists for non-insular species. Data points represent species, slopes represent linear models with standard error shaded. PGLS model results are reported. (C) Based on two-ratio and three-ratio models: insularity leads to elevated dN/dS ratios for short-branch species (dS < 0.1), while there is no impact of insularity for long-branch species (dS = 0.1–0.21). Parameter estimates and standard errors are shown. (D) dN/dS and island size show a far weaker relationship, which is not statistically significant. Points represent species, the slope represents a linear model with standard error shaded.

was sequenced from blood and is more at risk of including NUMTs, Supplementary Table 6). Assessing the interaction between dS and insularity using two-ratio and three-ratio models led to the same conclusions: when focusing on short branches (with dS < 0.1), there were significant differences in dN/dS between insular and non-insular species; but when focusing on longer branches (dS = 0.1–0.21), there was no such difference (Figure 2C).

Analysis of individual genes indicated that the tendency toward elevated dN/dS in insular species with short terminal branches was not driven by selection in any particular gene. Focusing on short-branch species, four of the five largest genes indicated greater dN/dS ratios in insular species (COX1: LRT = 5.21, p = .023, ND4: LRT = 6.07, p = .013, CYB: LRT = 3.59, p = .058, COX3: LRT = 8.13, p = .005, with ND1 providing the exception: LRT = 0.57, p = .45; Supplementary Figure 6A). Focusing on longer branches, none of the five largest genes showed elevated dN/dS in insular species (COX1: LRT = 0.68, p = .41, ND4: LRT = 1.96, p = .16, CYB: LRT = 0.07, p =

Table 1. Results of a phylogenetic generalized least squares model testing how the interaction between branch length (dS) and insularity affects dN/dS, in a model including tarsus length. N = 28 species (11 insular, 17 non-insular). λ = 0.082 (estimated by maximum likelihood).

Model term	Slope (SE)	*t*-value	*p*-value
dS: insularity (interaction)	−0.328 (0.103)	−3.17	.0043
dS	−0.367 (0.080)	−4.57	.0001
Insularity	0.055 (0.012)	4.62	.0001
log(tarsus length)	0.004 (0.007)	0.54	.6

Table 2. Results of a phylogenetic generalized least squares model testing how island size affects dN/dS among insular species, in a model including tarsus length and dS. $N = 11$ species. $\lambda = 0$ (estimated by maximum likelihood).

Model term	Slope (SE)	*t*-value	*p*-value
log(island size)	–0.003 (0.002)	–1.46	.19
log(tarsus length)	0.028 (0.014)	2.06	.079
dS	−0.365 (0.082)	−4.47	.0029

.79, ND1: LRT = 0.91, $p = .34$, COX3: LRT = 0.21, $p = .65$; Supplementary Figure 6B). Estimates of gene-specific dN/dS for the four smallest genes showed wider confidence intervals, but there remained a tendency for greater dN/dS in short-branch insular species, and no clear trend when focusing on longer branches (Supplementary Figure 6).

dN/dS is not related to island size

A phylogenetic *t*-test of log-transformed range size ~ insularity revealed that non-insular species occupy vastly larger geographic ranges than insular species ($F = 138.4$, $p < .0001$, Supplementary Figure 7A): insular mean back-transformed range size was 1,622 km^2 (95% CI: 348–7,567 km^2), vs. non-insular mean back-transformed range size 3,478,567 km^2 (95% CI: 1,856,549–6,517,699 km^2). However, dN/dS ratios within insular species were unrelated to island size, indicating that range limitations do not contribute to the high dN/dS ratios seen in insular rails (Figure 2D, Table 2). Tarsus length was not significantly larger in insular species (Supplementary Figure 7B), and had no significant impact on dN/dS in these models (Table 1, Table 2). Variance inflation factors indicated that collinearity between island size and tarsus size had little impact on the estimated effect of island size (vif ~1.6). An alternative model that used geographic range size instead of island size produced very similar results (Supplementary Table 7). We note that these models likely contain too many independent variables for the sample size ($n = 11$), however rerunning the models with only island size (or range size) as a predictor yielded similar results.

Weak purifying selection underlies the high dN/dS of recent colonizers

BUSTED analysis found that allowing site-specific dN/dS ratios to exceed 1 for short branches leading to insular species had little impact on the model fit (Log(L)=-89,758.82 vs. -89,760.09, $p = .14$), indicating that diversifying selection on a small number of sites was not responsible for the overall increase in dN/dS for this subset of branches. Further to this, dN/dS ratios > 1 were not more frequent in short branches leading to insular species than background branches (0.118% of sites for short insular branches vs. 0.139% of sites for background branches). In contrast, RELAX analysis found strong evidence that applying a generalized relaxation/weakening of selection along short insular branches (relative to short non-insular branches) led to an improvement of model fit (Log(L) –91,348.01 vs. –91,361.81, LRT $p < .0001$). This is consistent with small effective population sizes driving high dN/dS in recent island colonizers.

Discussion

Analysis of nine mitochondrial genes (8001 bp) and 40 rail species revealed that insular species exhibit elevated mitochondrial dN/dS due to the increased fixation of deleterious alleles, indicating a reduction in Ne consistent with studies in other avian clades (Brüniche-Olsen et al., 2019; James et al., 2016a; Johnson & Seger, 2001; Kutschera et al., 2020; Leroy et al., 2021; Recuerda et al., 2024; Stervander et al., 2015; Vlček et al., 2025). However, unlike some previous studies (Brüniche-Olsen et al., 2019; Gyllenhaal et al., 2025; Vlček et al., 2025), we found no evidence that range limitations due to island size are driving this pattern. Instead, we found that the signature of high dN/dS is limited to species with short terminal branches, i.e., recent colonizers, indicating that founder effects drive elevated dN/dS in island rails. This finding was highly consistent among different evolutionary models (free ratios, two-ratio vs. three-ratio, and RELAX), indicating that the results are not driven by a small subset of branches (a potential weakness of the two-ratio vs. three-ratio and RELAX approaches), that the results are not biased by overparameterization of short internal branches (a potential weakness of the free-ratios approach), and that the results are not driven by selection occurring on a handful of sites (a potential weakness of the free-ratios and two vs. three-ratio approaches).

A disadvantage of the dN/dS ratio used in this study is that the measure cannot easily separate the impacts of relaxed selection or positive selection from the impact of low effective population size. Since the insular rail populations analyzed here were also flightless, the increase in mitochondrial dN/dS seen in island rails could plausibly be driven by changes in the strength of selection on metabolic rates, rather than changes in Ne (Shen et al., 2009). However, this hypothesis is not strongly supported by previous research, with one previous study finding that slower-moving birds and mammals show elevated mitochondrial dN/dS ratios relative to faster-moving species (Shen et al., 2009), while another reported the opposite pattern of lower mitochondrial dN/dS in flightless lineages (Zwonitzer et al., 2023). Furthermore, in the context of the current study, relaxed (or positive) selection following flight loss would predict elevated dN/dS in both ancient and recent island colonizers, and may in fact predict a stronger signature in ancient colonizers, where species have been flightless for a greater proportion of the evolutionary time represented by the terminal branch. This prediction is inconsistent with the data, as the dN/dS of ancient colonizers was lower than that of recent colonizers and indistinguishable from volant relatives. A central role of positive selection would also predict particularly high dN/dS for specific genes or codons, which was not observed. The results are therefore most consistent with the founder effects hypothesis, and indicate that rails undergo a period of small population size during colonization that leads to the fixation of harmful alleles, but that populations subsequently recover, and the signature of inefficient purifying selection is lost due to a longer period of lower dN/dS (consistent with James et al., 2016a).

The results described here contrast sharply with those revealed by previous work on white-eye island colonizations, where initial founder effects appear minor compared with the long-term consequences of island range-limits (Clegg et al., 2002; Gyllenhaal et al., 2025; Sendell-Price et al., 2021). One key difference between the white-eye and rail systems

may relate to sociality. White-eyes are highly gregarious and are thought to colonize islands in large flocks of 48–357 individuals (Sendell-Price et al., 2021), which may blunt the initial costs of founding and lead to a more rapid recovery of effective population size. In contrast, rails are typically solitary or live in small family groups (Del Hoyo et al., 1996), which may lead to far smaller founding populations. Consistent with this interpretation, a human-mediated introduction of only ~17 white-eye individuals to Tahiti led to more severe losses in genetic diversity (Sendell-Price et al., 2021). Similarly, a within-species comparison of (volant) buff-banded rail *G. philippensis* populations found that smaller archipelagos hold less genetically diverse populations, indicating that range-limitation effects can be detected when gene flow precludes strong founder effects (Garcia-R et al., 2017).

Previous investigations finding that island population size variation is driven by range limitations in birds have also focused on far smaller islands than the current study (most islands were <5,000 km^2 in previous studies, whereas 50% of the islands in the current study are >10,000 km^2) (Brüniche-Olsen et al., 2019; Gyllenhaal et al., 2025; Vlček et al., 2025). Therefore, it may be that island size variation is influential when islands are very small but has little impact on larger scales. Further whole-genome-based work may yet reveal such patterns in rails, as the species that occupied the smallest islands in this dataset (the Wake island rail *G. wakensis* and the Laysan Island rail *Z. palmeri*) have accrued too few substitutions to accurately estimate species-specific dN/dS based on mitochondrial genes alone (fewer than 100 substitutions separate these species from their closest flying relatives).

Studies of avian vagrancy indicate that many potential colonization events do not give rise to enduring populations (Lees & Gilroy, 2014; Elliott, 1957). Since the present study only included "successful" species (those that are extant or very recently became extinct), there is a potential survivorship bias that must be considered when interpreting the pattern of results. Firstly, in theory, this bias could lead to a pattern of greater dN/dS ratios in shorter branches emerging in the absence of founder effects, if higher dN/dS populations go extinct before they reach the age that lower dN/dS populations can attain. However, such an explanation would predict a reasonably high frequency of low dN/dS recent colonizers, which was not observed. Secondly, survivorship bias may result in an underestimate of the strength of founder effects for island rails—if colonization success depends on founders being sufficiently numerous to avoid stochastic extinction or mutational meltdown, then the populations sampled here may only represent those with particularly weak founder effects. Similarly, only islands large enough to hold enduring rail populations were included in the study, and so the impact of extremely limited ranges may also be underestimated. Despite being limited in its application to unsuccessful colonizations, the study provides new insights into the drivers of variation in selection efficiency among extant and recently extinct island rail populations.

Islands are hotspots of extinction (Fernández-Palacios et al., 2021), and among island species, flightlessness makes rails particularly vulnerable, with 98%–99% of Pacific species lost since the start of the Holocene (Steadman, 2006), and just 40 island-limited species remaining worldwide today (Lévêque et al., 2021). Understanding the evolutionary processes that affect island rails may help to conserve the surviving populations (Balmford, 1996). To this end, the current study highlights that island rails may carry excess genetic load due to the severe bottleneck of effective population size associated with island colonization, which may leave them less fit and more susceptible to further perturbation (Briskie & Mackintosh, 2004; Frankham et al., 1999). However, there is no evidence that island rail populations continue to exhibit signatures of inefficient purifying selection once established (even on very small islands). This suggests that the accumulation of further genetic load and mutational meltdown is unlikely in the absence of further bottlenecks, and conserving the remaining rail populations on small islands is therefore a worthwhile endeavor. Note, however, that further work using nuclear genomes is needed to confirm the generality of this pattern, as mitochondrial genes exhibit unusually high mutation rates and are under unusually strong selective constraints (Neiman & Taylor, 2009; Popadin et al., 2012). More widely, this study indicates that the processes underlying signatures of low insular effective population size may vary according to details of the colonization process, leading to different patterns in different taxonomic groups.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

All gene alignments and scripts used in this analysis can be found at 10.5281/zenodo.18866525. All other data and the details of accession numbers for mitochondrial genomes can be found in Supplementary Table 1.

Author contributions

K.W. and P.A.H. conceptualized the study; P.A.H., M.S., G.R.G., S.D., D.B.Ø., G.C., S.F., G.Z., and K.W. generated the new mitochondrial data; R.T.K., E.L.B., and K.W. produced the mitochondrial gene alignments. K.W. and Z.O. performed all modeling and statistical analyses; K.W. wrote the manuscript; all authors contributed to manuscript edits and improvements.

Funding

P.A.H. and C.R. were supported by Villum Foundation Villum Investigator (grant no. 25925 to C.R.); P.A.H., K.W., and Z.O. were supported by Independent Research Fund Denmark (no. 1054-00039B). M.S. acknowledges support from a Marie Skłodowska-Curie fellowship (H2020-MSCA-IF-2019-893225). R.T.K. and E.L.B. were supported by the US National Science Foundation (DEB-1655683). S.F. and G.Z. were supported by the National Natural Science Foundation of China (grant nos. 32422009 to S.F. and 32388102 to G.Z.), S.F. was additionally supported by the National Key Research and Development Program of China (grant no. 2023YFA1800500 to S.F.), and G.Z. was additionally supported by the Villum Foundation (Villum Investigator grant no. 25900 to G.Z.).

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Emily Gordon and Elisabeth Smith for assistance in assembling mitogenomes. We also thank the museums and institutions that provided new material for sequencing: The Natural History Museum of Denmark (NHMD), the Natural History Museum in London (NHMUK), the Smithsonian National Museum of Natural History (USNM), the American Museum of Natural History (AMNH), the Field Museum of Natural History (FMNH), the Australian National Wildlife Collection (ANWC), the Natural History Museum in Oslo (NHMO), the Steinhardt Museum of Natural History (SMNHTAU), the Yale Peabody Museum (YPM), and the National University of Singapore (NUS). Finally, we thank the reviewers and the editors for their insightful comments and suggestions.

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Received October 8, 2025; revisions received April 2, 2026; accepted April 7, 2026
Associate Editor: Yvonne Willi; Handling Editor: Luis-Miguel Chevin