Rapid loss of flight in the Aldabra white-throated rail

Janske van de Crommenacker[1,2*], Nancy Bunbury[1,3], Hazel A. Jackson[2], Lisa J. Nupen[4], Ross Wanless[4,5], Frauke Fleischer-Dogley[1], Jim J. Groombridge[2,†] & Ben H. Warren[6,†]

[1] Seychelles Islands Foundation (SIF), La Ciotat Building, PO Box 853, Mont Fleuri, Victoria, Mahé, Seychelles

[2] Durrell Institute of Conservation and Ecology (DICE), School of Anthropology and Conservation, University of Kent, Marlowe Building, Canterbury, Kent CT2 7NR, UK

[3] Centre for Ecology and Conservation, University of Exeter, Cornwall Campus, Penryn, TR10 9FE, UK

[4] DST/NRF Centre of Excellence at the Percy FitzPatrick Institute of African Ornithology, University of Cape Town, Rondebosch 7701, Cape Town, South Africa

[5] Institute of Marine Affairs and Resources Management, National Taiwan Ocean University, Keelung, Taiwan

[6] Institut de Systématique, Evolution, Biodiversité (ISYEB), UMR 7205 CNRS MNHN UPMC EPHE UA, Muséum National d'Histoire Naturelle, Sorbonne Universités, 57 Rue Cuvier, 75005 Paris, France

* Corresponding author: janskevandecrommenacker@gmail.com

† Jointly senior (last) author

Short title: Rapid evolution of flightlessness in the Aldabra rail

Abstract

Flight loss has evolved independently in numerous island bird lineages worldwide, and particularly in rails (Rallidae). The Aldabra white-throated rail (*Dryolimnas* [*cuvieri*] *aldabranus*) is the last surviving flightless bird in the western Indian Ocean, and the only living flightless subspecies within *Dryolimnas cuvieri*, which is otherwise volant across its extant range. Such a difference in flight capacity among populations of a single species is unusual, and could be due to rapid evolution of flight loss, or greater evolutionary divergence than can readily be detected by traditional taxonomic approaches. Here we used genetic and morphological analyses to investigate evolutionary trajectories of living and extinct *Dryolimnas cuvieri* subspecies. Our data places *D.* [*c.*] *aldabranus* among the most rapid documented avian flight loss cases (within an estimated maximum of 80,000–130,000 years). However, the unusual intraspecific variability in flight capacity within *D. cuvieri* is best explained by levels of genetic divergence, which exceed those documented between other volant taxa versus flightless close relatives, all of which have full species status. Our results also support consideration of *Dryolimnas* [*cuvieri*] *aldabranus* as sufficiently evolutionary distinct from *D. c. cuvieri* to warrant management as an evolutionary significant unit. Trait variability among closely related lineages should be considered when assessing conservation status, particularly for traits known to influence vulnerability to extinction (e.g. flightlessness).

Key words

Aldabra Atoll, evolutionary significant unit, extinction, rapid evolution of flightlessness, isolated island population, Rallidae, taxonomic status

Introduction

Organisms living in island environments frequently undergo remarkable evolutionary changes [1–4]. One such change is loss of flight, which has occurred worldwide in 26 bird families from 17 orders [5]. Flight enables organisms to disperse, escape from predators and forage [e.g., 6,7]. Species-poor islands that naturally lack mammal and bird predators have been important in the evolution of flightlessness [8,9]. Consequently, loss of flight has evolved independently in many insular bird species worldwide. Despite the high incidence of avian flight loss on islands [9], the pace of evolutionary transitions underlying this trait is poorly known. This is at least partly due to the fact that many insular flightless or poorly volant bird species are extinct, and the scarcity of cases [11,12,13] in which there exist gradations in flightlessness among or within extant lineages.

The avian family with the highest incidence of flight loss worldwide is the Rallidae (rails; Order Gruiformes), with over 25% of the extant rail species being flightless [13]. The family includes an estimated 135–150 extant species, plus numerous extinct forms [14], with a global distribution that includes many oceanic islands, and a high proportion of island endemics [15].

Flightlessness has contributed to high extinction rates of island birds in the last 50,000 years, primarily driven by human colonization and the concomitant introduction of non-native predators [16]. Rallidae have probably been the most susceptible avian family in this regard. At least 65 species of Rallidae worldwide are documented as late Quaternary extinctions [17,18] and another 35 species as recent extinctions (since *ca.* 1500 years BP). However, it is estimated that such documented cases are greatly outnumbered by undocumented human-induced rail extinctions, which may total 2000 species in the Pacific islands alone [16,19]. Appropriate conservation assessment and protection of the remaining flightless Rallidae and other avian species is therefore vital.

Our research focuses on the last surviving flightless bird in the biodiversity hotspot of the Western Indian Ocean [20,21]: the Aldabra white-throated rail (*Dryolimnas* [*cuvieri*] *aldabranus*), which occurs only on Aldabra Atoll in the southern Seychelles. Historically, *D. cuvieri* occurred on all four islands of the Aldabra group – Aldabra, Assumption (Fig 1), Cosmoledo and Astove – before being extirpated from the latter three [10, 22–24]. There are two other recognised subspecies: the volant Madagascar white-throated rail *D. c. cuvieri*, a common endemic to Madagascar [10,22], and the extinct Assumption rail (*D. c. abbotti*), endemic to Assumption [25,26]. A second, extinct species of *Dryolimnas*, *D. augusti*, was recently described based on fossil remains from Réunion Island [27], and a third species, flightless and now extinct, once occurred on Mauritius [28,29]. Based on existing knowledge and applying the common assumption that taxonomic status reflects genetic divergence, the flightless Aldabra rail subspecies represents an enigma – it is flightless, yet only considered a subspecies in an otherwise volant species. Therefore, either it would appear to be a candidate for the youngest documented fully flightless bird lineage worldwide (and potential example of such an evolutionary change being very rapid; [28]), or it is more divergent from the Madagascar lineage than is readily inferred from current taxonomy.

Here we use genetic data from modern samples and museum specimens to examine the phylogenetic placement of the flightless lineages of the Aldabra group, and investigate whether or not their closest relative is indeed *Dryolimnas* of Madagascar. We further use these data, in combination with morphological data from modern and museum samples, to assess the degree of divergence of the flightless *D.* [*c.*] *aldabranus* and the poorly volant *D. c. abbotti* from the volant lineage of Madagascar. Genetic variation among populations of *D.* [*c.*] *aldabranus* is used to refine our understanding of important dispersal events in the biogeographic history of this lineage. We also show how differentiation among *D.* [*c.*] *aldabranus* subpopulations can be used to inform effective management of this unique bird, the last survivor among 12-17 flightless avian lineages that once occupied the Western Indian Ocean region before human arrival [24].

Materials and methods

Ethics statement

The ethical guidelines promoted by the Association for the Study of Animal Behaviour were followed. Permission for sampling on Aldabra was issued by the Seychelles Islands Foundation (local management authority), and the Department of Environment and the Seychelles Bureau of Standards approved all research activities (approval reference A0347). Sequences have been submitted to the NCBI GenBank (Accession Numbers: MH614934–MH614960, MH645373–MH645415 and MH651394–MH651440).

Study site and species

The total population of *D.* [*c.*] *aldabranus* occurs in an area of *ca.* 37.2 km^2, on the raised atoll of Aldabra (152.6 km^2, 9°24' S, 46°20' E; Fig 1): with subpopulations on Picard (area: 9.4 km^2), Polymnie (1.9 km^2) and Malabar (25.9 km^2). A UNESCO World Heritage Site since 1982, Aldabra has been managed entirely for research and conservation since 1979 with only a very small resident human population.

The Picard subpopulation of *D.* [*c.*] *aldabranus* originates from a successful reintroduction of 18 rails from Malabar in 1999 [30], after introduced feral cats on Picard were removed by humans in the 1970s [30]. For the sake of clarity regarding origin, we refer hereafter to these recently translocated rails on Picard with the term 'Malabar*'. This subpopulation has since expanded to more than 2500 individuals [31]. *Dryolimnas* [*c.*] *aldabranus* also occurred until very recently on the smaller islet of Île aux Cèdres, and was reportedly more morphologically distinct (leg and bill size) from *D.* [*c.*] *aldabranus* on other islands than was *D. c. abbotti* [23,25]. A recent extensive survey (Seychelles Islands Foundation (SIF), unpubl. data) indicated that this subpopulation is probably extinct (last confirmed sighting in 2000; Wanless, pers. obs.). The original *D.* [*c.*] *aldabranus* subpopulations on Grande Terre and Picard were extirpated (*ca.* mid-1800s and *ca.* 1910, respectively) following the introduction of feral cats [29,30, but see 24].

Dryolimnas c. abbotti was historically common on Assumption (~11 km^2; Fig 1), and was also well on its way to becoming flightless (i.e. being poorly volant, [22]), but had become extinct by 1937 [23,34,35], presumably due to the introduction of mammalian predators [11,30].

The volant *D. c. cuvieri* of Madagascar shows a stable population trend over its *ca.* 854,000 km^2 range [36], and is considered common [37], although no reliable population estimates are available. It occupies various habitats throughout Madagascar, including forest, wetlands, mangroves, beaches and rice paddy-fields [38].

Sample collection

Thirty-eight samples (S1 Appendix), representing all three *Dryolimnas* subspecies were analysed (including 19 historical toe pad samples from museum specimens, and 19 contemporary blood samples from living birds): 25 *D.* [*c.*] *aldabranus* samples (six historical, 19 contemporary), four *D. c. abbotti* (all historical), and nine *D. c. cuvieri* from different locations in Madagascar (all historical). The samples include individuals from all *D.* [*c.*] *aldabranus* subpopulations, except the extinct Grande Terre subpopulation, for which no museum specimens exist. Specimens from the extinct Picard subpopulation were available from museum skins. The 19 contemporary *D.* [*c.*] *aldabranus* blood samples were collected on Aldabra (Polymnie: *n* = 7, Malabar: *n* = 5, Malabar*: *n* = 4 and Île aux Cèdres: *n* = 3) in two periods (years 2000 [Île aux Cèdres] and 2011–2014). We used only historical *D. c.*

cuvieri samples after attempts to obtain contemporary samples were unsuccessful (i.e., despite several requests to different local researchers, nobody could provide us with samples).

DNA isolation, amplification and sequencing

DNA was extracted (S2 Appendix) using a Bioline Isolate Genomic DNA extraction kit (Bioline, UK), following the manufacturer's standard protocols for blood (contemporary samples) and tissue (museum samples). The museum samples had a range of ages dating back to the 1870s (S1 Appendix), and potentially low endogenous DNA concentration. They were therefore treated in a dedicated museum DNA laboratory. From each sample, 593bp from the mitochondrial regions Control Region (CR; 306bp) and Cytochrome b (Cytb; 287bp) was amplified and sequenced (Table 1, S2 Appendix). Negative controls were included to check the absence of contamination during the extraction and PCR process. For historical samples, amplifications were conducted using a suite of short overlapping fragment primers designed for this study with the NCBI Primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Table 1). PCR products were sequenced by Macrogen-South Korea and Macrogen-Europe. Sequence reads were manually checked and then aligned and edited using the programme FINCHTV 1.4 (Geospiza), BIOEDIT 7.2.0 [39] and CODONCODE ALIGNER 4.2.4 (CodonCode Corporation, Dedham, MA). Consensus sequences were aligned using the programme CLUSTALX 2.1.12 [40], and the genes were concatenated using SEQUENCEMATRIX [41].

Data partition, model selection and phylogenetic inference

For the concatenated mitochondrial dataset (593bp), the program PARTITIONFINDER [43] was used to test the congruence of phylogenetic signal from the different genes and determine the optimal substitution models of nucleotide evolution for each partition, according to Bayesian information criteria (BIC). The HKY + gamma evolutionary model was found to be the optimal model, and was used for the estimation of the time-calibrated phylogeny.

Molecular-based estimates of divergence: Time calibrated phylogenetic reconstruction

Time-calibrated phylogenies were reconstructed using BEAST v.1.8.2 [44] via the CIPRES Science Gateway [45]. Sequences from GenBank of Rallidae closely related to *Dryolimnas* – *Lewinia pectoralis*, *L. mirifica*, *L. muelleri*, *Gallirallus philippensis*, and two subspecies of *G. australis* – were selected as outgroups based on the phylogeny of Garcia-R *et al*. [46].

The following calibrations were specified: time to most common recent ancestor (TMRCA) of 2.588 Myr for the divergence of *Dryolimnas* and *Lewinia*, and 0.125 Myr for the most recent emergence of the Aldabra group. Our reasoning behind this choice of calibration dates was as follows:

1) The densely sampled phylogeny of Rallidae in Garcia–R. *et al*. [46] demonstrates that *Crex crex* shares a clade with *Lewinia* and *Dryolimnas* that gains 97% bootstrap support. Our phylogeny is fully congruent with that in Garcia–R. *et al*. [46]. A fossil *Crex crex* demonstrates that this taxon is at least 2.588 million years (Myr) old (http://fossilworks.org/bridge.pl). By deduction, the divergence of *Lewinia* and *Dryolimnas* in our tree must also be at least 2.588 Myr, and we calibrated it accordingly.

2) The estimated last emergence of the Aldabra group 0.125 ± 0.02 Ma ago [47] provided an upper bound estimate for the divergence of the common ancestor of *D. c. cuvieri* and the Aldabra group taxa (*D.* [*c.*] *aldabranus* and *abbotti*).

Some of the nodes we seek to date involve inter-specific relationships, while others may be intra-specific. Therefore, we compared results under the Yule speciation tree prior [51] with coalescent tree priors. Furthermore, we know that *D. cuvieri* has declined in population size (most severely on Aldabra) in historical times, but have no data on the nature of this decline. Therefore, under a coalescent tree prior we compared outputs with an inversegamma prior on population size dynamics, versus a uniform prior, assuming a constant unknown population size through time.

For each of the three alternative tree priors (Yule, Coalescent-Uniform, and Coalescent-Inversegamma), a lognormal relaxed clock was used with lognormal distributions for the calibration priors, and two replicate Monte Carlo Markov chains (MCMC) were performed for 10 million generations, sampling every 1000 generations under an HKY + gamma evolutionary model [48]. Mixing was confirmed by examining effective sample sizes (ESS>200) for all parameters using TRACER v1.6.0 [49]. Trees from the first 10% of generations were discarded as burn-in and a maximum clade credibility tree was summarised in TREEANNOTATOR v1.8.2 [44] and visualised in FIGTREE v1.4.2 [50]. After checking the convergence of Bayesian analyses through the congruence of outputs from replicate chains (under each alternative tree prior), two final MCMCs (Yule & Coalescent-Inversegamma tree priors) were performed for 30 million generations following the same protocol as for earlier chains.

Phylogenetic relationships: hypothesis testing

In addition to our Bayesian analyses, a best-scoring Maximum-Likelihood tree was reconstructed using RAXML v. 8.2.8 [51] under the GTR + G substitution model. Clade support was measured with the rapid bootstrap algorithm [52] using 5000 replicates. Furthermore, using the Shimodaira and Hasegawa (SH) test [53] implemented in PAUP*, we checked the monophyly of rail populations and discriminated between alternative scenarios of island colonization. Using the concatenated dataset, the SH test was used to compare the optimal Bayesian topology with topologies constrained to correspond to alternative hypotheses reconstructed using parsimony (heuristic searches, holding one tree at each step).

Haplotype networks

Median-joining haplotype networks were constructed (POPART v1.7; [47]) both for the concatenated mtDNA dataset, and for each marker separately, using the setting epsilon = 0 (minimum spanning network).

Morphological analyses

Morphological measurements (wing and tail length [using a flat ruler], tarsus length, bill length [bill tip to nasofrontal hinge], bill width and height [both measured at centre of nostrils]) were taken from all live birds and museum specimens. However, museum specimens tend to shrink upon drying [55] which compromises their reliability for comparison with live birds [56]. Therefore, only measurements from museum specimens were used for our morphological analyses. Measurements from museum specimens that were not genetically sampled were included to increase the sample size. To identify morphological differentiation between subspecies, a discriminant function analysis was performed in SPSS v25 (IBM). All traits were analysed separately with general linear models, with subspecies and sex as factors in the model. As a test for the homogeneity of slopes, the interaction between subspecies and sex were tested. Stepwise elimination was performed when the interaction and sex were found to be non-significant.

Results

Phylogenetic relationships, divergence times and genetic distances

Tree topology is highly concordant between Bayesian and ML analyses, between Bayesian analyses with different tree priors, and among replicate Bayesian analyses with the same tree prior. Bayesian analyses converged, with date estimates for supported (PP≥0.95) ingroup nodes varying by a maximum of 2.6% (1600 years) between replicate chains. Based on Bayesian analyses of 10 million generations, divergence time estimates show consistent variation depending on the tree prior used (Yule estimates being older than Coalescent-Uniform estimates, and Coalescent-Uniform estimates being older than Coalescent-Inversegamma estimates). We therefore selected Yule and Coalescent-Inversegamma tree priors for our final two Bayesian analyses (each was run for 30 million generations), thereby obtaining longer chains for the two tree priors that cover the full range of divergence estimates based on findings from shorter chains. Our Bayesian analyses (Fig 2) confirm that the flightless and poorly volant taxa of the Aldabra group (*D.* [*c.*] *aldabranus* and *D. c. abbotti* subspecies, respectively) are indeed most closely related to the volant white-throated rail of Madagascar (*D. c. cuvieri*; PP=1.0 for the monophyly of *D. cuvieri*). Although phylogenetic relationships are congruent with existing taxonomy in this respect, levels of genetic divergence are somewhat incongruous, with substantial genetic divergence within *Dryolimnas cuvieri;* the highest absolute sequence divergence of 2.1% between flightless *D.* [*c.*] *aldabranus* and volant *D. c. cuvieri* in Cytb, and 1.7% divergence between *D.* [*c.*] *aldabranus* and poorly volant *D. c. abbotti*, exceeds the minimum absolute divergence of 1.2% in Cytb encountered between other flightless and volant rail taxa, all of which have full species status [42]. Mean nucleotide divergences between the *Dryolimnas* subspecies derived from the concatenated (593 bp) mtDNA dataset are noteworthy in three cases (Table 2) between: 1) *D.* [*c.*] *aldabranus* and *D. c. cuvieri*; 2) *D. c. abbotti* and *D.* [*c.*] *aldabranus*; and 3) *D.* [*c.*] *aldabranus* populations native to the southern islands of Aldabra (Île aux Cèdres and Picard; putatively-extinct and extinct populations; herein “Native South Aldabra”) and *D.*

[*c.*] *aldabranus* populations native to northern islands of Aldabra (Malabar, Malabar* and Polymnie; extant; herein "Native North Aldabra").

Our relaxed clock analysis suggests that the divergence of Aldabra and Assumption populations from those on Madagascar occurred *ca.* 0.07–0.13 Myr ago. The Assumption population (*D. c. abbotti*) forms a monophyletic group within the species *Dryolimnas cuvieri* (>95% posterior probability under both Yule & Coalescent-Inversegamma tree priors). Tree topology is consistent with a lack of monophyly for all other subspecies; e.g., *D.* [*c.*] *aldabranus* populations from Malabar, Malabar* and Polymnie ("Native North Aldabra"; Fig 1) do not form a monophyletic group with *D.* [*c.*] *aldabranus* on Île aux Cèdres and Picard ("Native South Aldabra"; Fig 1) in any of the Bayesian analyses, nor in our ML analysis. However, all the relevant nodes lack significant branch support (i.e., ≥70% bootstrap values, ≥95% posterior probability regardless of tree prior). The SH test did not allow us to reject hypotheses of monophyly for each of the three major *D. cuvieri* populations: Aldabra group (i.e., Aldabra and Assumption), p=0.19; Aldabra, p=0.17; and Madagascar, p=0.18). Therefore, signal in our CR and Cytb data neither provides significant support for nor against the monophyly of these populations – both scenarios remain plausible.

Haplotype networks

Haplotype networks (Fig 3, S4 Appendix) show substantial genetic variation of *D. cuvieri* within the Aldabra group. *Dryolimnas c. cuvieri* of Madagascar is intermediate between two groups of *D.* [*c.*] *aldabranus* on each side of the network. This pattern in the concatenated mtDNA network (Fig 3) reflects divergence in the CR, rather than in Cytb (S4 Appendix). Distinct from *D. c. cuvieri* specimens is a major haplotype grouping represented mostly by contemporary specimens of *D.* [*c.*] *aldabranus* from Native North Aldabra (Fig 3). Another major grouping consists of historical *D.* [*c.*] *aldabranus* specimens from Native South

Aldabra, with *D. c. abbotti* between these haplotypes and those of Madagascar (Fig 3). The haplotype networks also indicate that *D. c. abbotti* has undergone fewer mutational changes relative to the Madagascar population than any of the *D.* [*c.*] *aldabranus* subpopulations.

Morphological analyses

Discriminant function analysis revealed the presence of morphological differences between *D. c. cuvieri, abbotti* and *aldabranus* (Wilks' lambda = 0.066, Chi-squared = 141.11, df=10, p<0.001; Fig 4). Two discriminant functions were found accounting for 100% of variation, with the first function accounting for 98.7% of variation between groups. Overall, the proportions of individuals correctly classified into their original groups were *D. c. cuvieri* = 96.3%, *D. c. abbotti* = 100% and *D.* [*c.*] *aldabranus* = 92.3%. The wings and tail of *Dryolimnas* [*c.*] *aldabranus* are the shortest, followed by *D. c. abbotti* and *D. c. cuvieri*, respectively. *Dryolimnas* [*c.*] *aldabranus* has a significantly longer bill than the other two subspecies (Table 3).

Discussion

Dryolimnas is a rare example of ability and inability to fly within what is currently considered a single species [23]. Our results suggest that the flightless *Dryolimnas* [*c.*] *aldabranus* has undergone an extended period of evolution on Aldabra (accumulating up to 2.1% absolute divergence from the Madagascar population, over an estimated 80,000–130,000 years). Loss of flight must have evolved rapidly, in less than 130,000 years based on our estimations, which concords with inferences made from subfossils [28]. This places the Aldabra rail well within the league of most rapid documented flight loss cases [8,13,15,42]. However, the enigma presented by its flightlessness does not seem fully explained by the speed of flight loss alone: there appear to exist younger fully flightless bird lineages worldwide, whether we consider date estimates alone (the flightless *Porzana palmeri* is estimated to have diverged within the past 125,000 years from its volant sister species, *Porzana pusilla*; [15]), or take genetic divergence as a proxy for time (flightless *Rallus sylvestris* showing only 1.2% absolute divergence in Cytb from volant *Rallus philippensis* [42]). Rather, the existence of a flightless (and poorly volant) subspecies within an otherwise volant species is primarily accounted for by the taxonomic status assigned to these taxa. To our knowledge, all other flightless bird lineages whose closest relatives are volant currently have full species status, even though the degree of genetic divergence encountered is sometimes lower (e.g. the *Rallus sylvestris-philippensis* case above) than the highest absolute divergences encountered here, of 2.1% between flightless *D.* [*c.*] *aldabranus* and volant *D. c. cuvieri*, and 1.7% between *D.* [*c.*] *aldabranus* and poorly volant *D. c. abbotti*.

The rapid evolutionary change associated with such cases of flight loss, despite low genetic divergence, is generally believed to be driven by selection rather than genetic drift, as maintaining such traits as energetically costly flight muscles [11,58] is presumably unnecessary in an environment in which the ability to fly confers little or no selective advantage [5,15,42). Indeed the energetic savings (and fat storage) associated with reduced flight musculature could be an adaptation to survive periods of food and water scarcity in Aldabra's long dry season [11]. Hume *et al.* [28,59] propose that *D.* [*c.*] *aldabranus* was already flightless by 100,000 ybp, as a fossil *D.* [*c.*] *aldabranus* tarsometatarsus from this period (found on Point Hodoul, Grande Terre) measures within the size range of the present flightless population of *D.* [*c.*] *aldabranus*. Flightlessness may result from variations in development of several physical traits [60]), such as underdeveloped pectoral muscles, asymmetry of wings (both confirmed to be the case for *D.* [*c.*] *aldabranus* [see 12]), increases in body mass, and changed proportions in skeletal elements [60,61). Changes in skeletal elements and body mass, associated with the evolution towards flightlessness, may also be present in the subspecies of *D. cuvieri,* but this remains to be tested. Mass differences were not possible to examine using museum skin specimens. Flightlessness can also be associated with shortened flight feathers (i.e, reduced wing and tail length [9,62]. Our finding that *D.* [*c.*] *aldabranus* has shorter wings and tail than *D. c. abbotti* and *D. c. cuvieri* supports reports from Ridgway and Abbott [26]) and Benson [22], but not Wanless [11]. Bill size may also evolve due to changes in foraging ecology [e.g., 56] and the longer bill of *D.* [*c.*] *aldabranus* (see below), also found by Benson [22], might be an adaptation to foraging for crabs/prey in limestone crevices. Concomitant evolution of flightlessness potentially facilitated this adaptation, as weight restrictions became less critical with the loss of flight. Male *D. c. cuvieri* generally had a longer bill than females (independent-samples t-test; p=0.009), and a longer bill length of *D.* [*c.*] *aldabranus* than *D. c. abbotti* and *D. c. cuvieri* was found in both sexes (all p<0.006, except for male *D.* [*c.*] *abbotti* which showed a borderline difference of p=0.07 with *D. c. aldabranus*).

Morphological changes are frequently due to selection on a limited number of loci. In the flightless Galapagos cormorant (*Phalacrocorax harrisi*), a series of candidate function-altering genetic variants was found that likely contributed to the evolution of flightlessness [60]. Given the gradations of rapid evolution towards flightlessness (and genetic differentiation) documented here in *Dryolimnas*, and the fact that both *D.* [*c.*] *aldabranus* and *D. c. cuvieri* are still extant, a genome-wide study should provide further insights into the adaptive evolution of flightlessness.

Colonisation patterns of D. [*c.*] *aldabranus*

Ancestors of *D.* [*c.*] *aldabranus* could have reached Aldabra via multiple colonisation events, which would explain the number of haplotypes within the living and historical populations of the Aldabra group relative to Madagascar, but is biogeographically puzzling.

Viewing the two main genetic groupings (Native South Aldabra and Native North Aldabra; Figs 2 and 3) as independent colonisations, it is curious that they have managed to remain separate lineages throughout the period since arrival. Aldabra has undergone numerous rapid and major changes in geography in the last 200,000 years, prior to the atoll's configuration today [59,64]. It may or may not have consisted of multiple islands at the time rails first colonised, and may have been a single island at least once since then. Regardless of precise history of changes in island geography and rail distribution, any scenario of two or more colonisations causing the genetic diversity of the Native South and North Aldabra populations we uncovered, needs to incorporate the inability of colonising populations to establish or introgress throughout the island or atoll, which is difficult to fully explain, assuming that at least one colonisation was of Madagascan origin, and fully volant upon arrival.

The alternative scenario of a single colonisation of Aldabra remains plausible given the lack of support for nodes generating the non-monophyly of the Aldabra populations (Fig 2), and the inability of the SH test to reject monophyly. For a single colonisation of the Aldabra group to explain the observed number and divergence of haplotypes, haplotype divergence of the small colonist population must have been as high, or higher, than it is across Madagascar today (Fig 3), at least for the CR (S4 Appendix). This, however, is not inconsistent with avian population histories in Madagascar. Humans arrived in Madagascar only 1500–2300 years ago [65], and have had a profound impact on native habitats [e.g., 66–68]. Recent (pre-human) avian extinctions and loss of genetic diversity in Madagascar have been speculated for various bird groups (see [69] for a review).

Whether one or multiple colonisations gave rise to *D. cuvieri* of the Aldabra group, the fact that rails native to South Aldabra are more closely related to those of Assumption than of North Aldabra (Fig 3, S4 Appendix) supports inter-island colonisation between Assumption and Aldabra. However, whether propagules from Madagascar colonised Aldabra via Assumption, or vice versa, is unclear.

Genetic differences of *D.* [*c.*] *aldabranus* between islands of Aldabra atoll itself are substantial, despite the lack of significant support for nodes in our data. It has been proposed that the restricted dispersal ability of *D.* [*c.*] *aldabranus* could limit gene flow between islands, resulting in inter-island genetic differences [30]. The probable genetic distinction of Île aux Cèdres rails from those on Malabar, Malabar* and Polymnie matches their distinctive morphological measurements [23,25] and plumage ([70], but differences were not observed by [25]). Furthermore, a high differentiation in microsatellites was found in rails on Île aux Cèdres and Polymnie, with respect to each other and to Malabar rails [30].

The separation of what are now the Native South and North Aldabra populations likely began when Aldabra presented a very different geographic setting from the one we know today, the present island configuration possibly being as recent as 5000–7000 years [59,64,71]. The isolation of the northern and southern islands of the atoll probably explains how the Native North and Native South lineages have remained isolated since then. Île aux Cèdres is a small (0.5 km^2) lagoon islet, closest to Grande Terre (distance: 253m) and separated from Malabar by a *ca.* 15m wide, deep channel (Fig 1). It is unlikely that flightless rails (at present sea level) would cross this channel. Île aux Cèdres' proximity to Grande Terre, where rails were presumably extirpated before the late 1800s, raises the possibility that these rails were a remnant of the extinct Grande Terre population. The fact that Île aux Cèdres rails cluster more closely to the original Picard rails than to those of other Aldabra islands appears counterintuitive as Picard lies on the other side of the atoll (Fig 1). However, the extinct Grande Terre rails may have resembled the extinct Picard rails, as the channels separating Picard and Grande Terre, are shallow (maximum 5m depth; [72]) and contain several islets, making gene flow between rails on these islands probable. In contrast, the channels between Grande Terre and Malabar (Passe Hoareau, *ca*. 15m depth), and between Picard and Polymnie (Main Channel, ca. 20m depth; [72]) are considerably deeper, with fewer ‘stepping stones’. Such barriers are expected to have maintained these populations isolated in recent times (<7000 ybp, and conceivably in earlier sea-level lowstands), with significantly reduced gene flow.

Our study provides a good example of the value of museum collections in understanding biogeographic and evolutionary history, and in informing conservation management of closely related extant species. Genetic and morphological data from museum specimens of extinct rail populations were essential to outline the evolutionary pathway of populations and identify appropriate conservation recommendations for *D.* [*c.*] *aldabranus*. Our understanding of extant genetic diversity would have been greatly impoverished without access to extinct genetic diversity archived in museum specimens.

Conservation management of D. [*c.*] *aldabranus*

Phylogenetic data, combined with data on morphology and behaviour, is a useful basis upon which to assess whether a population is sufficiently evolutionarily distinct from others to be treated as a separate conservation management unit. Despite morphological similarities between *Dryolimnas* on Aldabra and Madagascar, species boundaries have long been debated as it is argued that the populations must have been isolated for considerable time for flightlessness of the Aldabra population to have evolved. The surprisingly high genetic divergence and marked morphological differences of the Aldabra and Assumption subspecies from those of Madagascar, warrant the management, protection and assessment of the remaining Aldabra population as distinct from the Madagascar population. The small population size of *D.* [*c.*] *aldabranus* and its history of local extirpation, combined with the fact that it has evolved flightlessness and is consequently more vulnerable, increases the need for appropriate conservation management.

Dryolimnas cuvieri is currently Red-Listed as ‘Least Concern’ [73]. Unlike the common *D. c. cuvieri* on Madagascar, however, the restricted range, small population size and an ongoing threat from introduced cats on Grande Terre make *D.* [*c.*] *aldabranus* much more vulnerable to extinction. A Red List status that actually applies to a widely distributed, volant and less threatened subspecies is inappropriate and could compromise conservation management [74]. We therefore recommend re-assessment of *Dryolimnas cuvieri* subspecies by the IUCN to better reflect threat status. Given our results, *D.* [*c.*] *aldabranus* should at least be treated as a subspecies Vulnerable to extinction, based on IUCN criteria B and D2 (S5 Appendix).

Some authorities have already treated *Dryolimnas* [*c.*] *aldabranus* as a full species, distinct from *D. c. cuvieri* [e.g., 37]. The genetic divergence we uncover here certainly supports this view; to our knowledge, it is greater than that observed in all other such cases of closely-related volant-versus-flightless rail taxa, all of which are currently treated as full species. However, multiple species definitions are possible, with no single one being universally accepted [75,76]. Due to lack of significant support for nodes within *D. cuvieri*, our genetic data alone do not allow us to advocate treating *D.* [*c.*] *aldabranus* as a full species from a cladistic perspective. However, obtaining affordable and consistent sequence data from numerous historical samples necessarily restricted the length of sequence data obtained. It is conceivable that *D.* [*c.*] *aldabranus* will prove monophyletic based on genome-wide data, since our SH test showed that a hypothesis of monophyly cannot be rejected. Furthermore, regardless of whether or not *D.* [*c.*] *aldabranus* is monophyletic, it may well be a full species under a biological species concept. We remain open to such a decision being made by taxonomic authorities should they consider there to be sufficient justification.

In view of applying our results to conservation management and given the situation on the ground, we recommend the following conservation management measures:

1) Efforts to reinforce *D.* [*c.*] *aldabranus*' population should consider substantial genetic divergence between Native North and South Aldabra. Unfortunately, it is probable that the last remnant of the Native South Aldabra population (Île aux Cèdres) is now extinct. Nonetheless, it is possible that a few individuals are still present and, until this possibility is ruled out, translocation of individuals of Native North Aldabra origin to Île aux Cèdres (or Grande Terre) should be avoided. Performed prematurely, such a translocation risks extinguishing Native South Aldabra rail genetic diversity through hybridisation;

2) It is likely that the introduction of cats caused the extirpation of the original *D.* [*c.*] *aldabranus* subpopulations on Picard, Grande Terre and possibly also on Île aux Cèdres. Cats could easily colonise Aldabra's other islands from Grande Terre, so it is important to eradicate cats as soon as is logistically feasible on this large and remote island.

3) Rats may also compromise breeding success of *D.* [*c.*] *aldabranus*, although the effects may be limited (but not absent) as this species has been reported to be able to defend itself against, and even kill, rats [see 77]. Nevertheless, for broad conservation reasons, planning for a rat eradication programme is underway and should be prioritized; however, during eradication it will be essential to maintain a captive population of rails from as broad a geographic range as possible across Polymnie and Malabar to safeguard the genetic variation they present.

4) Translocation of *D.* [*c.*] *aldabranus* should be considered to other islands in the Aldabra group (e.g., Assumption) and Western Indian Ocean preferably only when rat- and cat-free. Translocated groups should contain individuals from both Polymnie and Malabar.

As the last extant flightless bird in the Western Indian Ocean, the Aldabra white-throated rail has unique conservation significance. Our research sheds new light both on the phylogeny and evolution of flightlessness in *Dryolimnas*, and on its colonisation history, with important implications for conservation management. The flightless *D.* [*c.*] *aldabranus* is clearly on a separate evolutionary trajectory from the volant *D. c. cuvieri*. Its evolutionary uniqueness, based on genetic and morphological divergence, warrants treating *D.* [*c.*] *aldabranus* as an independent conservation management unit.

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Tables

Table 1. Primers and experimental conditions used to amplify and sequence the genes (in contemporary and historical samples) used.

					PCR conditions			
			Contemporary specimens					
DNA type	Gene region	Primer names	Sequence 5' - 3'	Source	Nr of cycles	Denaturation	Annealing	Extension
Mitochondrial DNA	Cytochrome b	L14841	AAAAGCTTCCATCCAACATCTCAGCATGATGAAA	[42]	40	95°C for 15 sec	58°C for 15 sec	72°C for 10 sec
		H15156	AAACTGCAGCCCCTCAGAATGATATTT					
	Control Region	RailCRcompSPEC-f	GCGTACCCCCTACTTTCAAGG	Own design	33	95°C for 15 sec	56°C for 15 sec	72°C for 10 sec
		RailCRcompSPEC-r	GACCGAGGAACCAGAGGC					
			Historical specimens					
	Gene region	Primer names	Sequence 5' - 3'	Source	Nr of cycles	Denaturation	Annealing	Extension
Mitochondrial DNA	Cytochrome b	Cytb/1 (f & r) (96 bp)	GCACTACACTGCAGACACAA (f) & TTAGCGTGGAGGTTGCGG (r)	Own design	35	95°C for 15 sec	55°C for 15 sec	72°C for 10 sec
		Cytb/2 (f & r) (114 bp)	CACATGCCGCAACGTACAAT (f) & GAGCCGTAGTAGAATCCTCGG (r)					
		Cytb/3 (f & r) (132 bp)	GCCGAGGATTCTACTACGGCTC (f) & CCCCTCAGAATGATATTTGTCCTCA (r)					
	Control Region	For *D.* [*c.*] *aldabranus* and *abbotti*: RailCRcompSPEC (f & r) (351 bp)	See modern DNA primer	Own design	35	95°C for 15 sec	58°C for 15 sec	72°C for 10 sec
		For *D. c. cuvieri*: MadRailCR (f & r) (351 bp)	See modern DNA primer	Own design	35	95°C for 15 sec	58°C for 15 sec	72°C for 10 sec

* All PCR amplifications were started with an initial denaturation step of 1 min at 95°C before commencing the cycles.

Table 2. Divergences of the different populations/(sub)species of *D. cuvieri* for Cytb and CR combined, and for Cytb alone. The genetic distance metric used is absolute distance.

Comparison of *D. cuvieri*	**Gene**	**Pairwise substitutions**	**Genetic distance**
D. [*c.*] *aldabranus* vs *D. c. cuvieri*	Cytb-CR	3–9	0.51–1.5%
	Cytb alone	0–6	0–2.1%
D. c. abbotti vs *D. c. cuvieri*	Cytb-CR	4–8	0.67–1.3%
	Cytb alone	0–1	0–0.35%
D. [*c.*] *aldabranus* vs *D. c. abbotti*	Cytb-CR	2–9	0.34–1.5%
	Cytb alone	0–5	0–1.7%
D. [*c.*] *aldabranus*: Île aux Cèdres from Native North Aldabra (Malabar-Malabar*-Polymnie)	Cytb-CR	3–9	0.51–1.5%
	Cytb alone	0–7	0–2.4%
D. [*c.*] *aldabranus*: Picard (extinct) from Native North Aldabra	Cytb-CR	3–6	0.51–1%
	Cytb alone	0–5	0–1.7%

Malabar* = Picard population recently introduced from Malabar

Table 3. Subspecies differences (between *D. c. cuvieri*, *abbotti* and *aldabranus*) for different morphological measurements.

		Mean ± SD			**Subspecies**			**Covariate: Sex**			**Sex * subspecies**		
Parameter	**Sex**	*D.[c.]aldabranus*	*D.c.abbotti*	*D.c.cuvieri*	*F*	*d.f.*	*p*	*F*	*d.f.*	*p*	*F*	*d.f.*	*p*
(a) Wing length (mm)	Male	116.66 ± 7.03 (n=15)	135.25 ± 2.06 (n=4)	154.43 ± 7.39 (n=14)	**177.58**	**2**	**<0.001**	0.51	1	0.48	**3.14**	**2**	**0.051**
	Female	118.58 ± 4.75 (n=13)	135.67 ± 2.89 (n=3)	147.67 ± 7.98 (n=15)									
(b) Tail length (mm)	Male	32.71 ± 5.77 (n=15)	54.16 ± 3.41 (n=5)	61.93 ± 5.33 (n=14)	**175.49**	**2**	**<0.001**	0.71	1	0.4	2.42	2	0.097
	Female	36.96 ± 5.32 (n=13)	57.8 ± 3.47 (n=3)	59.67 ± 7.26 (n=15)									
(c) Bill length (mm)	Male	45.79 ± 2.57 (n=15)	42.48 ± 3.16 (n=5)	42.0 ± 2.83 (n=14)	**15.77**	**2**	**<0.001**	**10.26**	**1**	**<0.001**	1.5	2	0.23
	Female	43.86 ± 2.83 (n=13)	37.0 ± 4.51 (n=3)	40.34 ± 2.32 (n=15)									
(d) Bill width (mm)	Male	5.94 ± 0.47 (n=15)	6.06 ± 0.49 (n=5)	5.94 ± 0.58 (n=15)	1.66	2	0.2	**8.88**	**1**	**0.004**	2.38	2	0.1
	Female	5.28 ± 0.73 (n=13)	5.27 ± 0.15 (n=3)	5.85 ± 0.44 (n=14)									
(e) Bill height (log) (mm)	Male	8.93 ± 0.90 (n=14)	9.53 ± 0.54 (n=4)	9.47 ± 0.70 (n=15)	2.34	2	0.1	3.12	1	0.08	0.16	2	0.86
	Female	8.52 ± 0.91 (n=12)	8.8 ± --- (n=1)	9.2 ± 0.74 (n=15)									

Table legend: (A) wing length, (B) tail length, (C) bill length, (D) bill width and (E) (log)bill height, with sex analysed as covariate (along with the interaction between subspecies and sex). The values shown are results from final models where the subspecies*sex and sex were eliminated respectively, if non-significant (statistically significant parameters are shown in bold).

Figures

Fig 1. (A) Western Indian Ocean with Madagascar, Aldabra Atoll and Assumption Island (the latter two enlarged in the inset), and (B) the islands of Aldabra Atoll, of which Picard, Malabar and Polymnie are populated by *D.* [*c.*] *aldabranus*, as was Île aux Cèdres until recently.

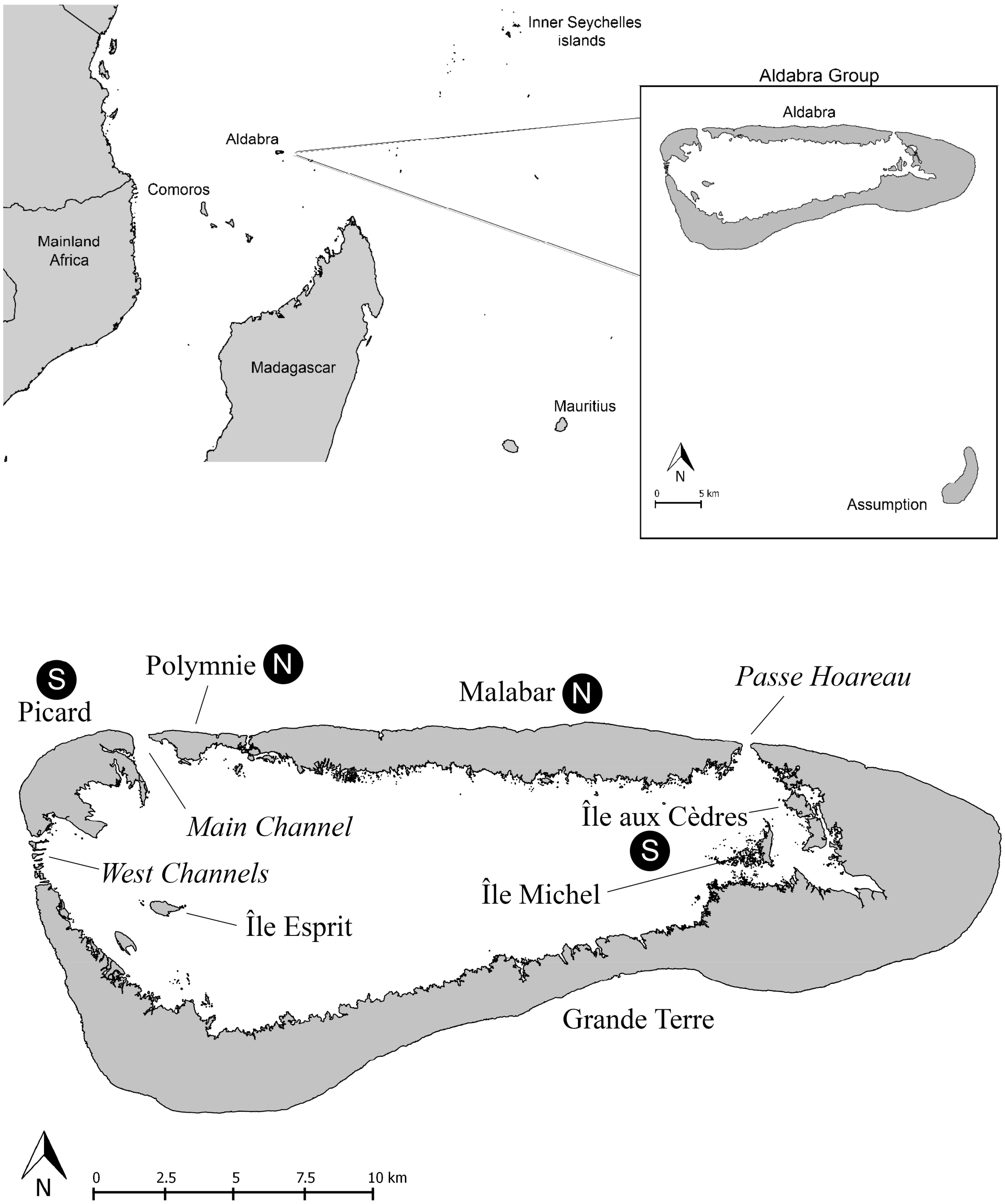


Fig 2. Bayesian analysis (Yule speciation prior, 30 million generations) of concatenated Cytb and CR mtDNA data from contemporary and museum (indicated with ^) specimens of *D. c. cuvieri* from Madagascar, *D. c. abbotti* from Assumption, and *D.* [*c.*] *aldabranus* from Aldabra (different islands; indicated with colours, and Native North (N) and South (S) Aldabra islands are indicated with the black encircled letters). Bayesian branch support values (>75%) are indicated. Error bars display the 95% higher posterior density and time on the x-axis is given in millions of years before the present. († = population now extinct, Mlb* = Picard population recently introduced from Malabar). Although the analysis with the Yule speciation prior was illustrated here because of the interspecific nature of our deeper-level sampling (see [57] for discussion), the equivalent analyses with Coalescent-Inversegamma and Coalescent-Uniform speciation priors are illustrated in Appendix S3. Furthermore, to magnify nodes and confidence intervals of interest for our focus, we excluded the outgroups from this figure. The full tree (Yule speciation prior) including the outgroups can also be found in Appendix S3.

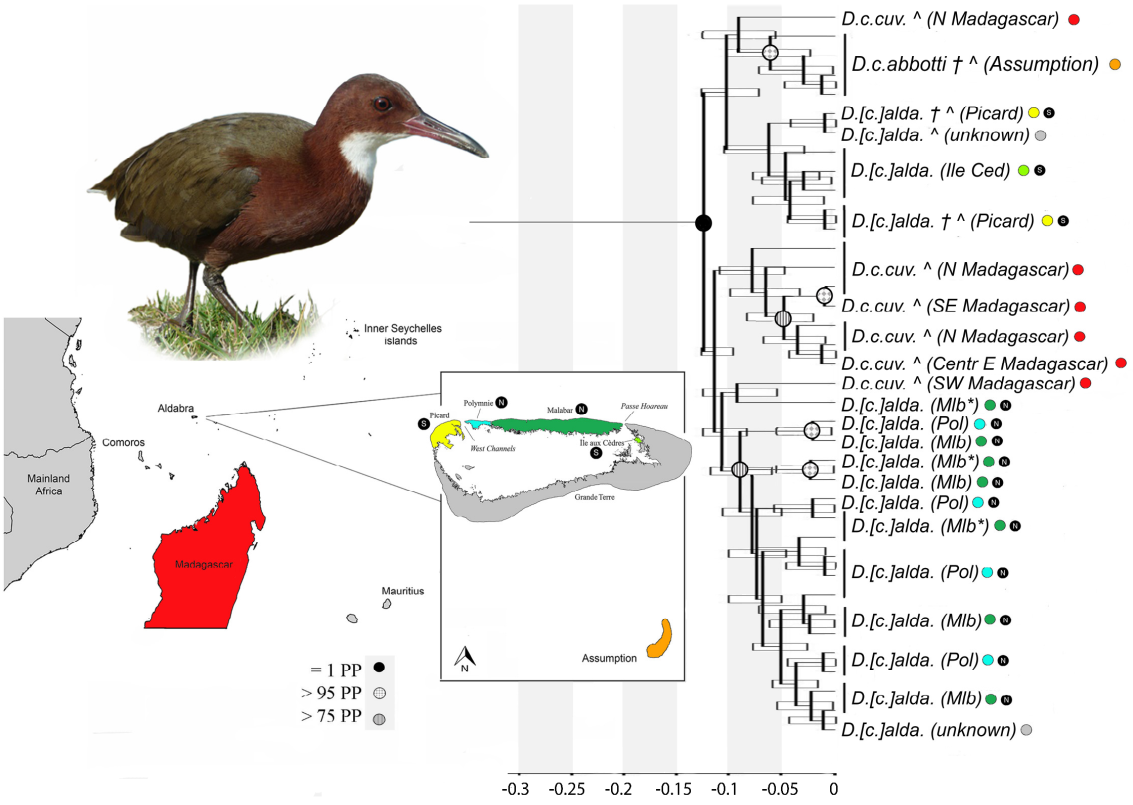


Fig 3. Median-joining haplotype networks for concatenated mtDNA (including CR and Cytb). For the Aldabra rail, the individuals from Malabar and Malabar* are pooled. Native North (N) and South (S) Aldabran islands are indicated with the encircled letters. Median-joining haplotype networks for each of the separate markers can be found in S4 Appendix.

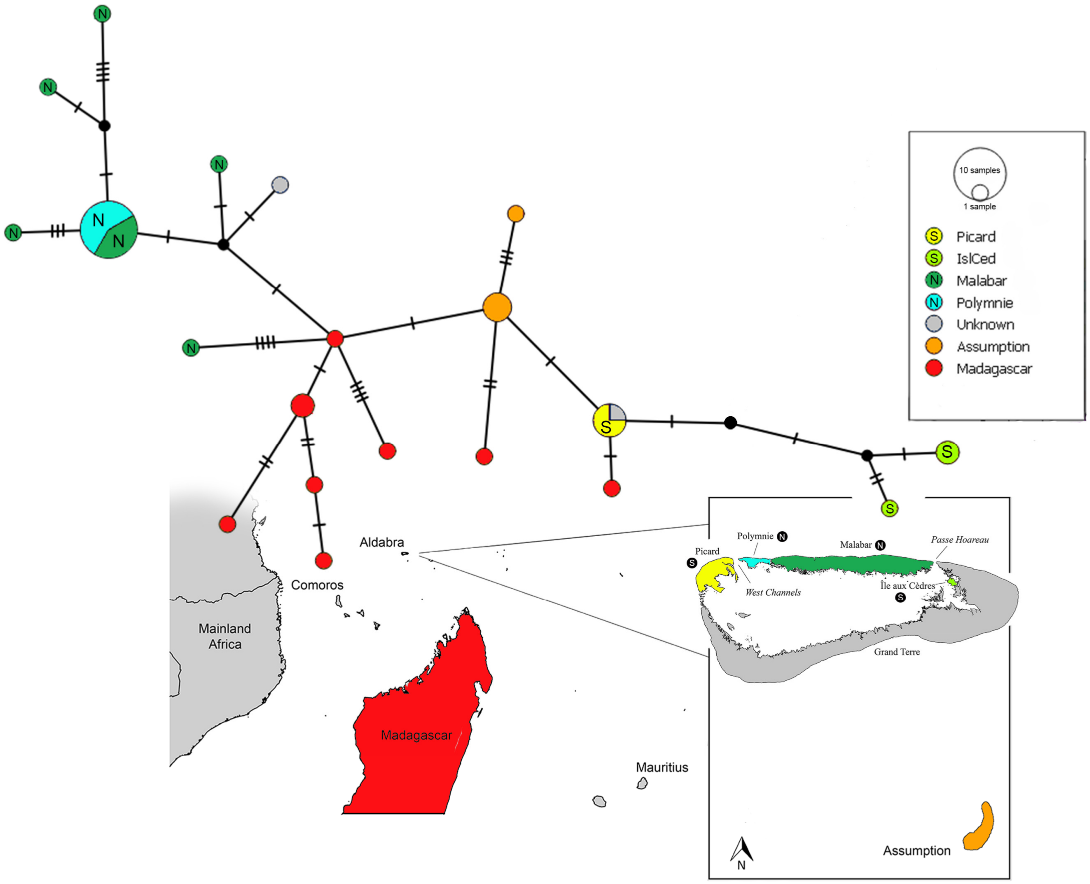
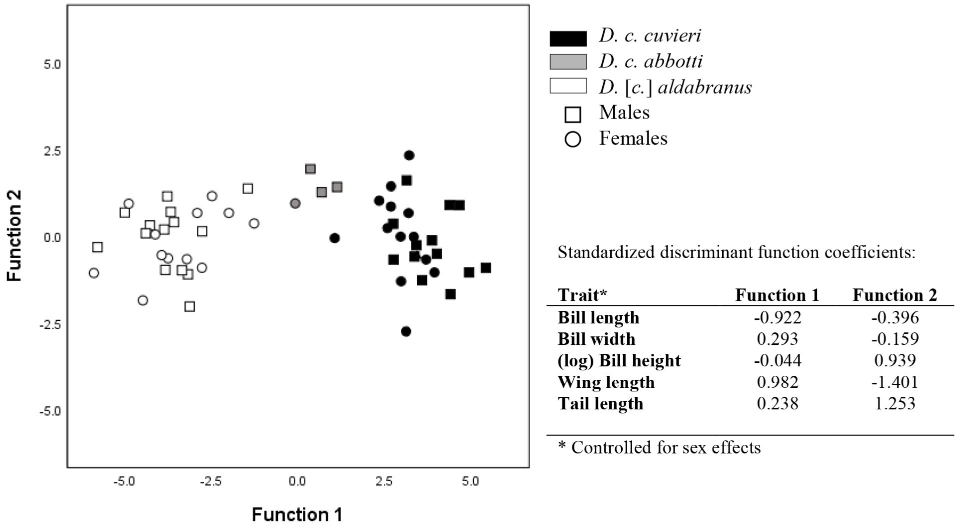


Fig 4. Plot of the two canonical functions resulting from the discriminant function analysis, with their coefficients for each of the morphological variables. Prior to the analysis, the data were corrected for sex. Different symbols indicate the different sexes.



Standardized discriminant function coefficients:

Trait*	**Function 1**	**Function 2**
Bill length	-0.922	-0.396
Bill width	0.293	-0.159
(log) Bill height	-0.044	0.939
Wing length	0.982	-1.401
Tail length	0.238	1.253

* Controlled for sex effects

Supporting information captions

S1 Appendix. Detailed information for the historical specimens used in this study.

S2 Appendix. Molecular methods.

S3 Appendix. (1) Phylogenetic tree from Fig 2 (Yule speciation prior, 30 million generations), with the outgroups included. (2) Dated cladogram applying Coalescent-Inversegamma speciation prior, 30 million generations. (3) Dated cladogram applying Coalescent-Uniform speciation prior, 10 million generations.

S4 Appendix. Median-joining haplotype networks for each of the markers used in this study.

S5 Appendix. Evaluation of *D.* [*c.*] *aldabranus* classification against IUCN criteria.

Supporting information

S1 Appendix. Detailed information for the historical specimens used in this study.

NHM = Natural History Museum in Tring, UK; AMNH = American Museum of Natural History in New York, USA); USNM = Smithsonian Institution, National Museum of Natural History, Washington DC, USA).

Subspp.	**Genetics ID (Fig. 2)**	**Museum Label**	**Collection date**	**Museum**	**Sample location**	**Lab ID (if included in genetic analyses)**	**Morpho-logical analyses?**
Samples included in this studies' genetic AND morphological analyses							
D. [*c.*] *aldabranus*	*D. [c.] aldabranus* M32	1968.43.102	12/03/1968	NHM	Aldabra Atoll, island unknown	AldaRail32	X
D. [*c.*] *aldabranus*	*D. [c.] aldabranus* M33	1906.12.28.14	10/1906	NHM	Aldabra Atoll, island unknown	AldaRail33	X
D. [*c.*] *aldabranus*	*D. [c.] aldabranus* M34	1977.10.70	18/05/1972	NHM	Aldabra Atoll, Malabar	AldaRail34	X
D. [*c.*] *aldabranus*	*D. [c.] aldabranus* M35	AMNH545395	14/10/1903	ANMH	Aldabra Atoll, Picard (pre-extinction)	AldaRail35	X
D. [*c.*] *aldabranus*	*D. [c.] aldabranus* M36	AMNH545396	14/10/1903	ANMH	Aldabra Atoll, Picard (pre-extinction)	AldaRail36	X
D. [*c.*] *aldabranus*	*D. [c.] aldabranus* M37	AMNH545397	14/10/1903	ANMH	Aldabra Atoll, Picard (pre-extinction)	AldaRail37	X
D. c. abbotti	*D. c. abbotti* 1	1906.12.21.139	12/03/1906	NHM	Assumption	AssRail1	X
D. c. abbotti	*D. c. abbotti* 2	1906.12.21.141	12/03/1906	NHM	Assumption	AssRail2	X
D. c. abbotti	*D. c. abbotti* 3	1906.12.21.142	12/03/1906	NHM	Assumption	AssRail3	X
D. c. abbotti	*D. c. abbotti* 5	1906.12.21.140	12/03/1906	NHM	Assumption	AssRail5	X
D. c. cuvieri	*D. c. cuvieri* 100	1931.8.18.1765	27/11/1930	NHM	N Madagascar, Bezona, East of Ambanja	MadRail100	X
D. c. cuvieri	*D. c. cuvieri* 101	1931.8.18.1004	02/11/1930	NHM	N Madagascar, Andranofanjava	MadRail101	X
D. c. cuvieri	*D. c. cuvieri* 102	1931.8.18.1759	24/11/1929	NHM	SW Madagascar, Befandriana	MadRail102	X
D. c. cuvieri	*D. c. cuvieri* 103	1931.8.1.8.1757	22/08/1930	NHM	N. Madagascar 1 Day West of Andapa	MadRail103	X
D. c. cuvieri	*D. c. cuvieri* 105	1969.48.101	n/a	NHM	Centr. Madagascar, District de Rogez, foret orientale	MadRail105	X
D. c. cuvieri	*D. c. cuvieri* 106	1889.11.3.72	3/3/1881	NHM	N. Madagascar, Tsikoza, Ankafana	MadRail106	X
D. c. cuvieri	*D. c. cuvieri* 107	1931.8.18.1002	13/08/1929	NHM	SE Madagascar, Ivohibe	MadRail107	X
D. c. cuvieri	*D. c. cuvieri* 108	1931.8.18.1760	26/10/1930	NHM	N. Madagascar, Mt. D'Ambre,	MadRail108	X
D. c. cuvieri	*D. c. cuvieri* 109	1931.8.18.1764	03/01/1931	NHM	N. Madagascar, Bezona, East of Ambanja	MadRail109	X
Samples NOT included in this studies' genetic analyses, but used for morphological analyses							

D. [*c.*] *aldabranus*	--	1939.12.9.2939	08/07/1906	NHM	Aldabra Atoll, specific location unknown (likely Picard)	X
D. [*c.*] *aldabranus*	--	1906.12.21.144	13/03/1906	NHM	Aldabra Atoll, specific location unknown (likely Malabar)	X
D. [*c.*] *aldabranus*	--	78.12.31.5	n/a	NHM	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	1906.12.21.143	15/03/1906	NHM	Aldabra Atoll, specific location unknown (likely Malabar)	X
D. [*c.*] *aldabranus*	--	1904.12.4.6	Sept-Nov 1906	NHM	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	1907.12.4.5	Sept-Nov 1906	NHM	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	1939.12.9.2938	08/07/1906	NHM	Aldabra Atoll, specific location unknown (likely Picard)	X
D. [*c.*] *aldabranus*	--	AMNH545384	08/07/1906	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545385	08/07/1906	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545386	08/07/1906	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545387	08/07/1906	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545388	n/a	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545389	n/a	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545390	n/a	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545391	n/a	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545392	n/a	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545393	01/10/1903	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545394	01/10/1903	ANMH	Aldabra Atoll, specific location unknown	X
D. [*c.*] *aldabranus*	--	AMNH545398	28/09/1903	ANMH	Aldabra Atoll, Picard	X
D. [*c.*] *aldabranus*	--	AMNH545399	02/09/1903	ANMH	Aldabra Atoll, Picard	X
D. [*c.*] *aldabranus*	--	AMNH545400	02/10/1903	ANMH	Aldabra Atoll, Picard	X
D. [*c.*] *aldabranus*	--	AMNH545401	14/10/1903	ANMH	Aldabra Atoll, Picard	X
D. [*c.*]	--	AMNH545402	14/10/1903	ANMH	Aldabra Atoll,	X

aldabranus					Picard		
D. [*c.*] *aldabranus*	--	USNM128833	04/10/1892	USNM	Aldabra Atoll, specific location unknown		X
D. [*c.*] *aldabranus*	--	USNM128830	01/10/1892	USNM	Aldabra Atoll, specific location unknown		X
D. [*c.*] *aldabranus*	--	USNM128834	10/10/1892	USNM	Aldabra Atoll, specific location unknown		X
D. [*c.*] *aldabranus*	--	USNM128837	18/10/1892	USNM	Aldabra Atoll, specific location unknown		X
D. [*c.*] *aldabranus*	--	USNM128836	18/09/1892	USNM	Aldabra Atoll, specific location unknown / Assumption?		X
D. c. abbotti	*D. c. abbotti* 4 (poor quality sequence)	1906.12.21.138	12/03/1906	NHM	Assumption	AssRail4	X
D. c. abbotti	--	USNM128827	18/09/1892	USNM	Assumption		X
D. c. abbotti	--	USNM128828	18/09/1892	USNM	Assumption		X
D. c. abbotti	--	USNM128829	18/09/1892	USNM	Assumption		X
D. c. cuvieri	*D. c. cuvieri* 104 (poor quality sequence)	1931.8.18.1761	24/11/1929	NHM	SW Madagascar, Befandriana	MadRail104	X
D. c. cuvieri	--	Unreg.	1888	NHM	Madagascar, specific location unknown		X
D. c. cuvieri	--	1879.6.7.6	n/a	NHM	Centr. Madagascar, Betsiles		X
D. c. cuvieri	--	Unreg.	1888	NHM	Madagascar, specific location unknown		X
D. c. cuvieri	--	1931.8.18.1000	07/06/1930	NHM	NE Madagascar, SW of Maroantsetra		X
D. c. cuvieri	--	1889.11.3.71	03/1881	NHM	N. Madagascar, Ankafana		X
D. c. cuvieri	--	1889.11.3.73	n/a	NHM	Madagascar, specific location unknown		X
D. c. cuvieri	--	1866.5.5.30	n/a	NHM	E. Madagascar, Mohambo		X
D. c. cuvieri	--	1866.5.5.25	n/a	NHM	E. Madagascar, Mohambo		X
D. c. cuvieri	--	1931.8.18.999	10/06/1930	NHM	NE Madagascar, SW of Maroantsetra		X
D. c. cuvieri	--	1891.8.1.82	n/a	NHM	Madagascar, specific location unknown		X
D. c. cuvieri	--	1882.2.27.112	14/03/1881	NHM	N. Madagascar, Ankafana		X
D. c. cuvieri	--	1931.8.18.1003	09/08/1929	NHM	SE Madagascar, Ivohibe		X
D. c. cuvieri	--	1891.8.1.80	20/10/1874	NHM	Madagascar, Mare du Vinang Sambyre(?)		X
D. c. cuvieri	--	1891.8.1.83	n/a	NHM	SE Coast Madagascar		X
D. c. cuvieri	--	1931.8.18.1767	26/03/1931	NHM	Centr. Madagascar, Ambararatabé		X
D. c. cuvieri	--	1843.7.22.69	n/a	NHM	Madagascar, specific location unknown		X
D. c. cuvieri	--	1931.8.18.1762	31/08/1930	NHM	N. Madagascar, West of Andapa		X
D. c. cuvieri	--	1890.10.16.330	n/a	NHM	Madagascar, specific location unknown		X
D. c. cuvieri	--	1969.43.39	n/a	NHM	Madagascar, specific location unknown		X
D. c. cuvieri	--	1969.52.1065	23/11/1886	NHM	NE Madagascar, Riviere Ivolina,		X

					Amajoustre	
D. c. cuvieri	--	1931.8.18.1763	01/05/1929	NHM	Centr. Madagascar, Foret Sianaka	X
D. c. cuvieri	--	1931.8.18.1758	13/08/1929	NHM	SE Madagascar, Ivohibe	X
D. c. cuvieri	--	1931.8.18.1001	17/08/1929	NHM	SE Madagascar, Ivohibe	X
D. c. cuvieri	--	1931.8.18.1766	23/01/1931	NHM	N. Madagascar, East of Maromandia	X

S2 Appendix.

Molecular methods

DNA extraction, PCR and Sequencing

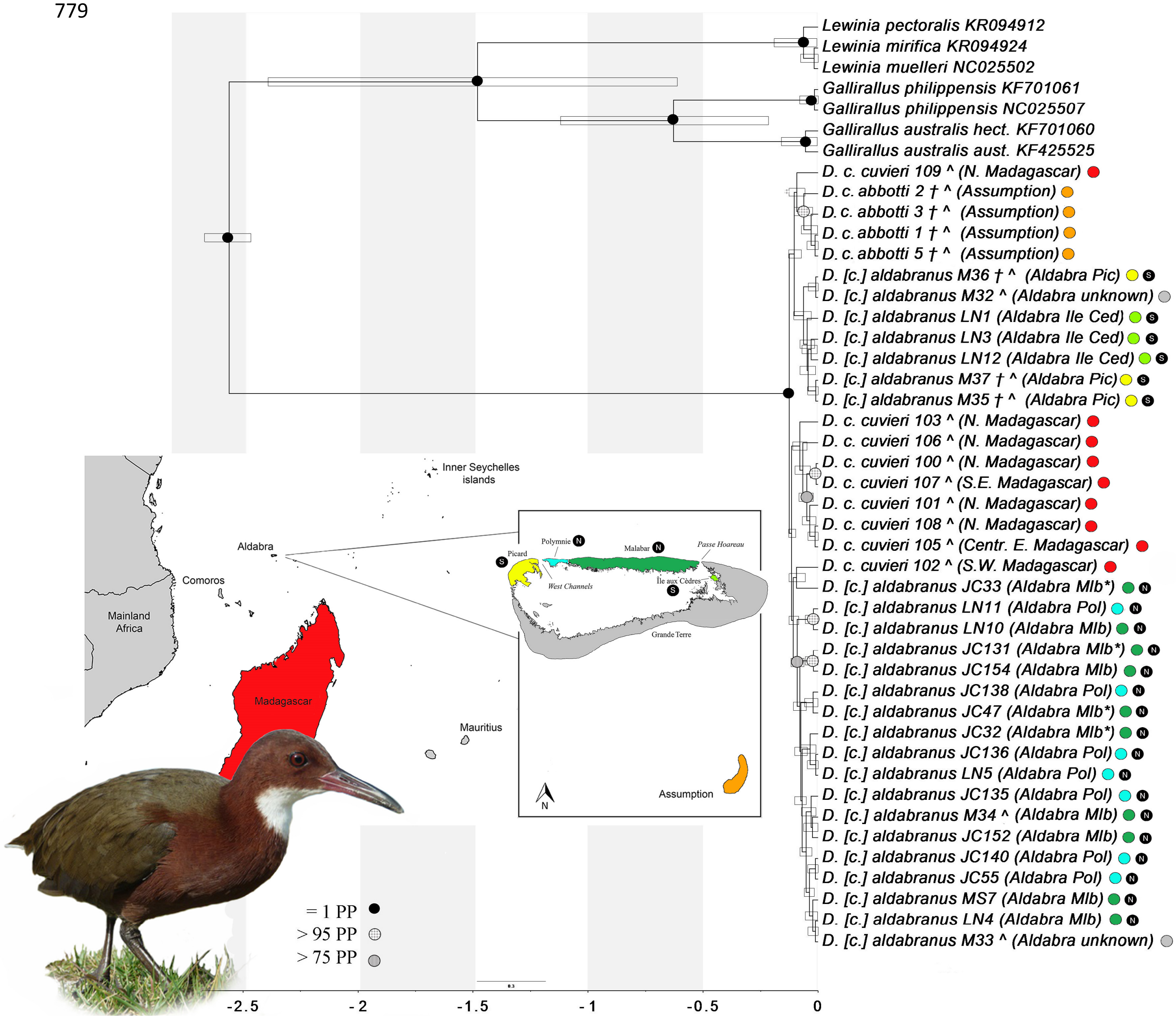
DNA from all blood samples and museum toepad specimens were extracted using the Isolate Genomic DNA Mini Kit (Bioline, UK). Samples were suspended in 400 µl Lysis buffer plus 40 µl (blood) or 25 µl (chopped museum toepads) of proteinase K and incubated at 55ºC overnight (or until the toepad material had completely digested). DNA was washed through a spin column and suspended in 200 µl (blood) or 50 µl (museum specimens) elution buffer. Typically, 25µl PCRs were prepared, comprising the following reagents: 1µl DNA extract, 2µl of each of the forward and reverse primers (at 10µM dilution), 12.5µl My Taq HS Red Mix (Bioline, UK) and 7.5µl UV sterilised DNA grade distilled water (dH_2O). PCR amplification of target regions was performed under the following cycling conditions: initial denaturation (1 min at 95°C); *n* cycles (marker-specific; Table 1) of 15 sec at 95°C, 15 sec at marker-specific temperature; Table 1, 10 sec at 95°C, and a final 10 min extension at 72°C. PCR results were verified by agarose gel electrophoresis with SybrSAFE staining and visualised using a Bio-Rad Gel Doc^{TM} EZ Imager (viewing software: Bio-Rad Image Lab 3.0).

PCR products were purified and sequenced by Macrogen (Europe and South Korea). Sequence reads were manually checked and edited using the programmes FINCHTV 1.4 (Geospiza), BIOEDIT 7.2.0 (69) and CODONCODE ALIGNER 4.2.4 (CodonCode Corporation, Dedham, MA). Consensus sequences were aligned using the programme CLUSTALX 2.1.12 (70).

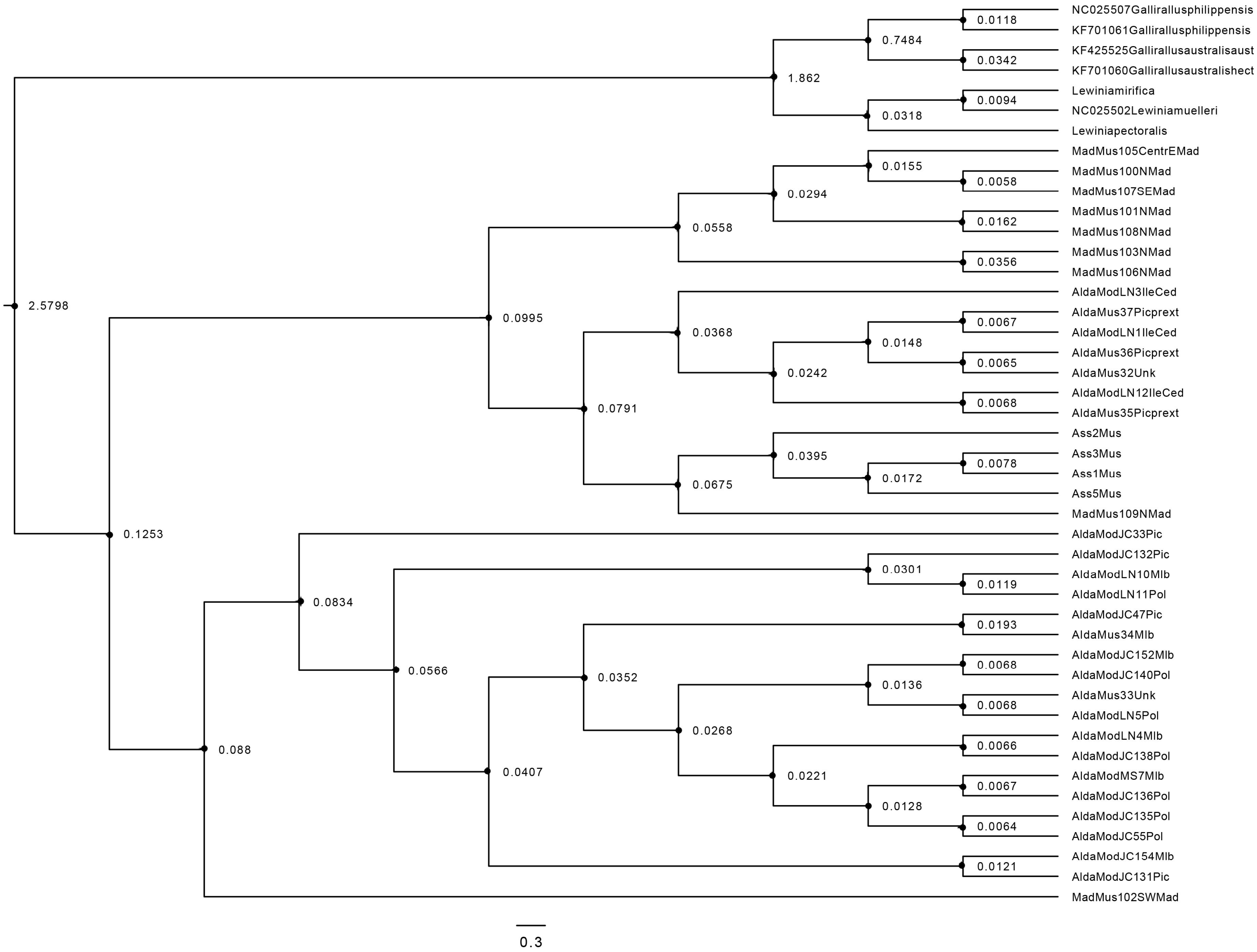
S3 Appendix.

(1) Phylogenetic tree from Fig 2 (Yule speciation prior, 30 million generations), with the outgroups included. (2) Dated cladogram applying Coalescent-Inversegamma speciation prior, 30 million generations. (3) Dated cladogram applying Coalescent-Uniform speciation prior, 10 million generations.

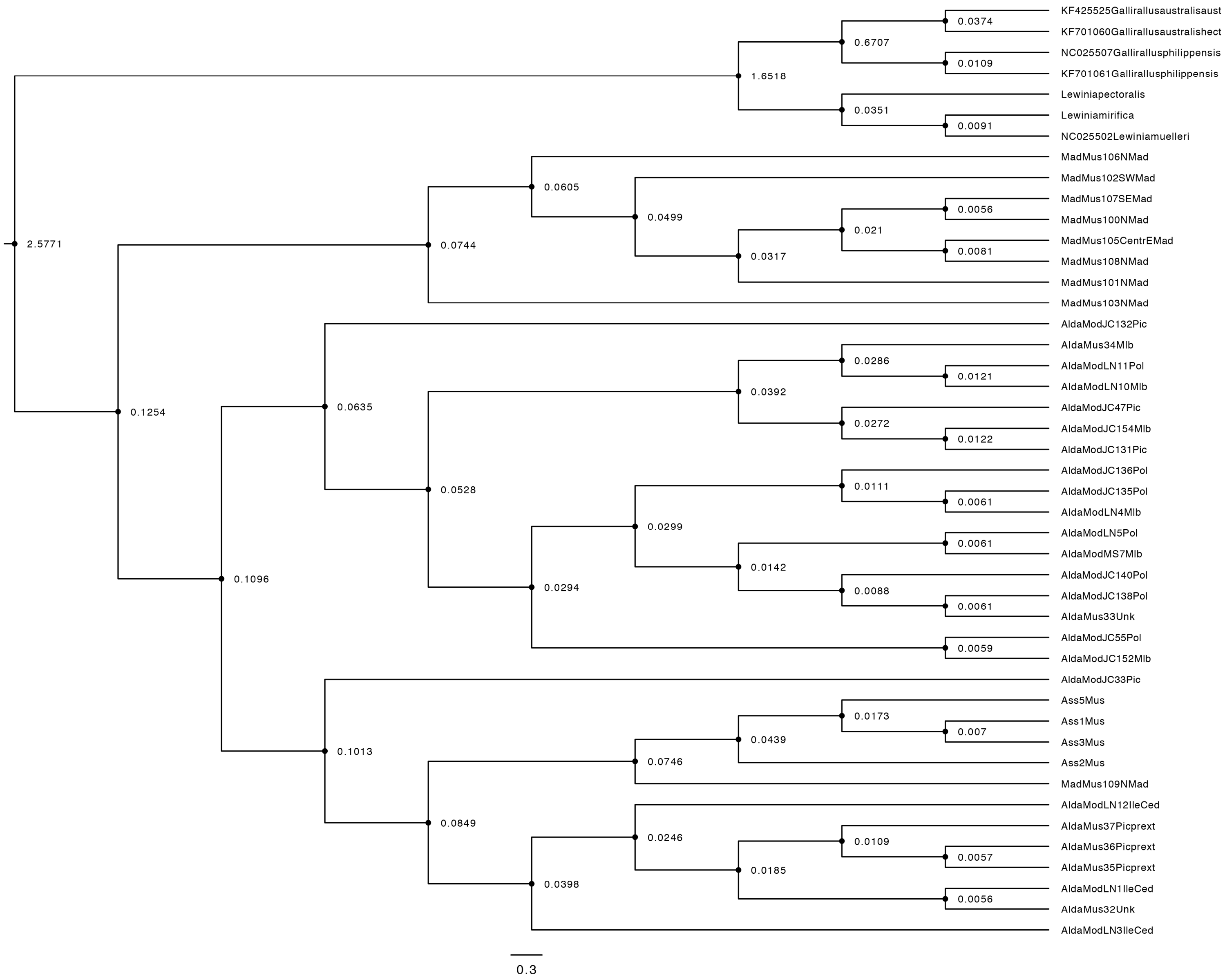
(1) Phylogenetic tree Figure 2



(2) Dated cladogram applying Coalescent-Inversegamma speciation prior



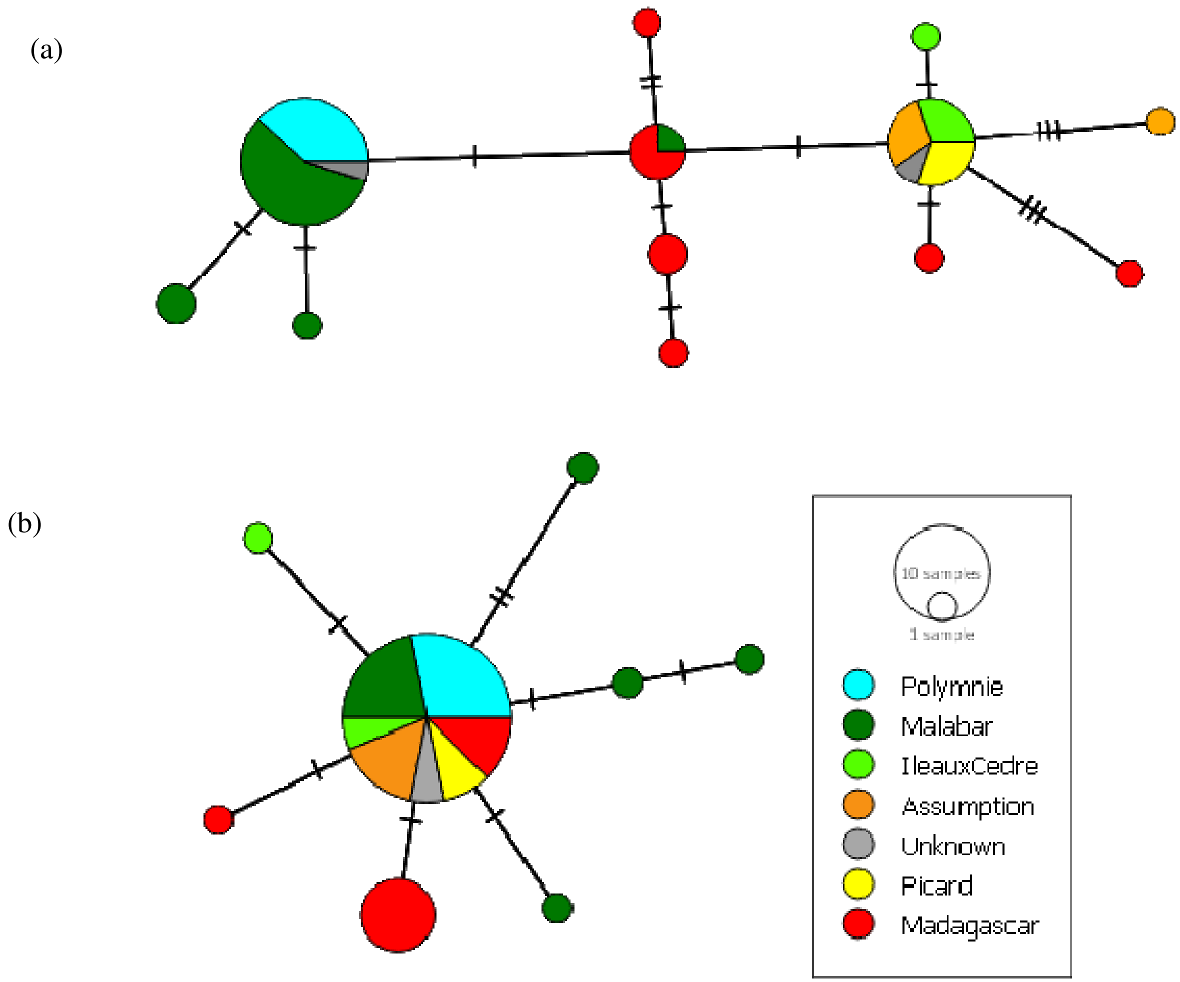
(3) Dated cladogram applying Coalescent-Uniform speciation prior



S4 Appendix.

Median-joining haplotype networks for each of the markers used in this study.

(a) Control Region and (b) Cytb. For the Aldabra rail, the Picard pre-extinction individuals, those from Île aux Cèdres and those caught from unknown locations are shown separately, whereas the individuals from Malabar and Malabar* are pooled.



S5 Appendix.

Evaluation of *D.* [*c.*] *aldabranus* classification against IUCN criteria

Dryolimnas [*c.*] *aldabranus* is now classified by IUCN as being Least Concern. Classification within this category means that it has been evaluated against the IUCN criteria and does not qualify for Critically Endangered, Endangered, Vulnerable or Near Threatened. Widespread and abundant taxa are included in the category of Least Concern.

We propose *D.* [*c.*] *aldabranus* to be treated as Vulnerable. Classification in this category applies when the best available evidence indicates that it meets any of the following criteria (A to E), and it is therefore considered to be facing a high risk of extinction in the wild:

A. Reduction in population size based on any of the following:

1. An observed, estimated, inferred or suspected population size reduction of ≥50% over the last 10 years or three generations, whichever is the longer, where the causes of the reduction are clearly reversible AND understood AND ceased, based on (and specifying) any of the following:

(a) direct observation
(b) an index of abundance appropriate to the taxon
(c) a decline in area of occupancy, extent of occurrence and/or quality of habitat
(d) actual or potential levels of exploitation
(e) the effects of introduced taxa, hybridization, pathogens, pollutants, competitors or parasites.
Not applicable

2. An observed, estimated, inferred or suspected population size reduction of ≥30% over the last 10 years or three generations, whichever is the longer, where the reduction or its causes may not have ceased OR may not be understood OR may not be reversible, based on (and specifying) any of (a) to (e) under A1.
Not applicable

3. A population size reduction of ≥30% projected or suspected to be met within the next 10 years or three generations, whichever is the longer (up to a maximum of 100 years), based on (and specifying) any of (b) to (e) under A1.
Not applicable

4. An observed, estimated, inferred, projected or suspected population size reduction of ≥30% over any 10 year or three generation period, whichever is longer (up to a maximum of 100 years in the future), where the time period must include both the past and the future, AND where the reduction or its causes may not have ceased OR may not be understood OR may not be reversible, based on (and specifying) any of (a) to (e) under A1.
Not applicable

In general, the population of *D.* [*c.*] *aldabranus* on Aldabra is currently considered to be stable. The reintroduced population on Picard has expanded to more than 2500 individuals since 1999 [1]. However, a subpopulation of *D.* [*c.*] *aldabranus* has most

likely gone extinct recently on Île aux Cèdres, which was estimated to be at least 80 individuals in the mid-1970s [2,3]. *Dryolimnas* [*c.*] *aldabranus* was last confirmed to be present on Île aux Cèdres in 2000, when Wanless took blood samples of birds there [4]. This reduction, potentially due to the arrival of introduced predators (cats) or decline of habitat quality due to extended drought, warrants listing as Vulnerable under this criterion.

<u>B. Geographic range</u> in the form of either B1 (extent of occurrence) OR B2 (area of occupancy) OR both:

1. Extent of occurrence estimated to be less than 20,000 km2, and estimates indicating at least two of a-c:
a. Severely fragmented or known to exist at no more than 10 locations.
b. Continuing decline, observed, inferred or projected, in any of the following:
(i) extent of occurrence
(ii) area of occupancy
(iii) area, extent and/or quality of habitat
(iv) number of locations or subpopulations
(v) number of mature individuals.
c. Extreme fluctuations in any of the following:
(i) extent of occurrence
(ii) area of occupancy
(iii) number of locations or subpopulations
(iv) number of mature individuals.
Yes – 1a and 1b(iv) are applicable

2. Area of occupancy estimated to be less than 2,000 km2, and estimates indicating at least two of a-c:
a. Severely fragmented or known to exist at no more than 10 locations.
b. Continuing decline, observed, inferred or projected, in any of the following:
(i) extent of occurrence
(ii) area of occupancy
(iii) area, extent and/or quality of habitat
(iv) number of locations or subpopulations
(v) number of mature individuals.
c. Extreme fluctuations in any of the following:
(i) extent of occurrence
(ii) area of occupancy
(iii) number of locations or subpopulations
(iv) number of mature individuals.
Yes – 2a and 2b(iv) are applicable. *Dryolimnas* [*c.*] *aldabranus* has an Extent of Occurrence of 37.2 km^2 (i.e., the islands Picard (9.4 km^2), Malabar (25.9 km^2), Polymnie (1.9 km^2) and a few satellite lagoon islets near Malabar) and meets the threshold for Endangered under criterion B1 (i.e., extent of occurrence estimated to be <100 km^2), and its Area of Occupancy meets the threshold for Endangered (<500 km^2) under criterion B2. Furthermore, the Île aux Cèdres subpopulation appears to have become recently extinct. The species' range is currently considered stable, but there is a high possibility of continuing decline in the future as a result of the potential impacts of climate change (increasing drought frequency, sea level rise), and invasive predators such as cats and rats, in particular the threat of cats establishing on other islands with

rails is very high. Additionally, it is likely found at less than five locations (see Criterion D). Therefore, it could potentially warrant listing as Endangered, or alternatively at least as Vulnerable under criteria B.

C. <u>Population size</u> estimated to number fewer than 10,000 mature individuals and either:

1. An estimated continuing decline of at least 10% within 10 years or three generations, whichever is longer, (up to a maximum of 100 years in the future) OR
No, but see threats mentioned below

2. A continuing decline, observed, projected, or inferred, in numbers of mature individuals AND at least one of the following (a-b):
a. Population structure in the form of one of the following:
(i) no subpopulation estimated to contain more than 1,000 mature individuals, OR
(ii) all mature individuals in one subpopulation.
b. Extreme fluctuations in number of mature individuals.
No

The population size of this species has been estimated at ca. 2500 birds on Picard [1]. Previously published estimates for the other islands are outdated: intensive studies in the 1970s yielded population estimates of 7700 rails on Malabar, 270 on Polymnie and 80 on Île aux Cèdres [2]. New estimates are underway, but it is anticipated that the total population size is approximately or less than 10,000 mature individuals. At the moment there is no indication for a continuing decline, but threats such as the arrival / spread of introduced predators, decline of habitat quality due to extended drought frequency, or habitat loss due to sea level rise warrant listing as Vulnerable under this criterion.

<u>D. Population very small or restricted</u> in the form of either of the following:

1. Population size estimated to number fewer than 1,000 mature individuals.
No

2. Population with a very restricted area of occupancy (typically less than 20 km2) or number of locations (typically five or fewer) such that it is prone to the effects of human activities or stochastic events within a very short time period in an uncertain future, and is thus capable of becoming Critically Endangered or even Extinct in a very short time period.
Yes

The population size of *D.* [*c.*] *aldabranus* is larger than the criterion of 1,000 mature individuals. However, the number of locations where *D.* [*c.*] *aldabranus* is found is very small (three locations covering 37.2 km^2), with subpopulations being confined to even smaller islands (i.e., the islands Picard (9.4 km^2), Malabar (25.9 km^2), Polymnie (1.9 km^2). It could be questioned whether Aldabra Atoll itself is considered to be one location or if the four main constituent islands with subpopulations present (Malabar, Picard and Polymnie) are considered separate locations. Based on the potential threats listed under Criterion C in combination with this limited range, *D.* [*c.*] *aldabranus* may qualify

as Vulnerable under criterion D2.

E. Quantitative analysis showing the probability of extinction in the wild is at least 10% within 100 years.

Criterion E – No quantitative analysis of extinction risk has been conducted for this species. Therefore, it cannot be assessed against this criterion.

Based on the above aspects, we propose *D.* [*c.*] *aldabranus* be up-listed to at least Vulnerable under criteria B and D2.

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1. Šúr M, van de Crommenacker J, Bunbury N. Assessing effectiveness of reintroduction of the flightless Aldabra rail on Picard Island, Aldabra Atoll, Seychelles. Conservation Evidence. 2013;10:80–4.
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