Ancient DNA Elucidates the Controversy about the Flightless Island Hens (Gallinula sp.) of Tristan da Cunha

Dick S. J. Groenenberg1*, Albert J. Beintema2, René W. R. J. Dekker1, Edmund Gittenberger1,3

1 National Museum of Natural History Naturalis, Leiden, The Netherlands, 2 Biowrite, Gossel, The Netherlands, 3 Institute of Biology, Leiden University, Leiden, The Netherlands

Abstract

A persistent controversy surrounds the flightless island hen of Tristan da Cunha, Gallinula nesiotis. Some believe that it became extinct by the end of the 19th century. Others suppose that it still inhabits Tristan. There is no consensus about Gallinula comeri, the name introduced for the flightless moorhen from the nearby island of Gough. On the basis of DNA sequencing of both recently collected and historical material, we conclude that G. nesiotis and G. comeri are different taxa, that G. nesiotis indeed became extinct, and that G. comeri now inhabits both islands. This study confirms that among gallinules seemingly radical adaptations (such as the loss of flight) can readily evolve in parallel on different islands, while conspicuous changes in other morphological characters fail to occur.

Citation: Groenenberg DSJ, Beintema AJ, Dekker RWRJ, Gittenberger E (2008) Ancient DNA Elucidates the Controversy about the Flightless Island Hens (Gallinula sp.) of Tristan da Cunha. PLoS ONE 3(3): e1835. doi:10.1371/journal.pone.0001835

Editor: Jean-Nicolas Volff, Ecole Normale Superieure de Lyon, France

Received October 3, 2007; Accepted February 17, 2008; Published March 19, 2008

Copyright: © 2008 Groenenberg et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: groenenberg@naturalis.nl

Introduction

Until recently it was assumed that the flightless moorhen of remote Tristan da Cunha in the southern Atlantic (Fig. 1), Gallinula nesiotis (Sclater, 1861) [1], became extinct by the end of the 19th century [2]. A few decades after its description, a very similar moorhen that was also flightless namely G. comeri (Allen, 1892), was described [3] from the island of Gough, ca. 400 km SE of Tristan. In the period between these descriptions G. nesiotis became rare [4,5] and by the turn of the century it had probably gone extinct [7,6]. Authentic remnants are two skins and a skeleton in the Natural History Museum, Tring [8]. Since unequivocal G. nesiotis had been collected only once from Tristan, and because of the presence of a healthy population of similar moorhens on the nearby island of Gough, some authors doubted whether an endemic moorhen had ever existed on Tristan [9]. Eber [10] compared Sclater’s description of G. nesiotis from Tristan with her series of G. comeri from Gough and concluded that the differences fall within the range of variation of the latter. In her opinion it was very unlikely that moorhens from two islands in the same region would have independently lost the ability of flight, without differentiating in other characters. She suggested that Sclater’s material might have been labelled inaccurately and that her specimens in fact also came from Gough. Consequently, Eber considered G. comeri a junior synonym of G. nesiotis [10] and controversy surrounded future illustrations of both taxa (Fig. 2).

Beintema [2] mentioned that there are old records of moorhens for Tristan, demonstrating that such birds were truly indigenous there. In his view, skeletal measurements differ slightly between G. nesiotis and G. comeri. Furthermore, rails are known to rapidly lose the ability of flight as soon as they arrive on remote islands [11]. When in 1972 live moorhens were discovered on Tristan [12], these birds were regarded as descendants of a small number of individuals brought from Gough [13]. Alternatively, Beintema suggested that G. nesiotis might have been temporarily rare on Tristan, but not extinct, and that the moorhens found there today are descendents of the original island population. Here we address this question, making use of DNA analyses of authentic material of G. nesiotis, recent specimens of the moorhens from both Tristan and Gough and some geographically and taxonomically close other taxa of moorhens.

Results

An overview of the specimens that were used in this study, with taxon names, locality data, and year of acquisition, is given in Table 1. Alignments of all cloned sequences of G. nesiotis (two independent amplifications per target region) are shown in Dataset S1, S2, S3. On the one hand, none of the sequences of genuine, historical G. nesiotis was identical to those of G. comeri and, on the other hand, all sequences of the moorhens collected in Tristan da Cunha in 1993, are identical to those found for specimens of G. comeri from Gough, dated 1960. The genetic distances between G. nesiotis and the other gallinules, are of the same magnitude as the distances between G. comeri and the other gallinules (Table 2). A pairwise relative rates test did not reveal significantly different substitution rates for any of the lineages (P > 0.10). Of the selected markers, most variation was detected in the control region (D-loop) sequences, viz. 9.6% TSH for G. nesiotis compared with G. comeri (= Total Sequence Heterogeneity [15,14]) versus 2.1% and 0.3% TSH for tRNA-Lys/ATP synthase subunit 8 (ATP8) and cytochrome b, respectively.

The results of phylogenetic analyses (Neighbour-Joining, Maximum Likelihood and Bayes) based on a combined dataset (all taxa, all regions, Dataset S4) are shown in Fig. 3–5. In these cladograms Gallinula nesiotis and G. comeri form a clade with the
moorhens of Africa/Eurasia, whereas the other taxa that were investigated are less closely related.

Discussion

Our results show that genuine *G. nesiotis*, identified on the basis of historical material from the island of Tristan da Cunha, differs genetically from *G. comeri*, which has been described from the island of Gough. For each marker the sequence of *G. nesiotis* differs from that of *G. comeri*, as well as from all other *Gallinula* taxa that were analysed, but the amount of variation differs strongly between the regions studied. The position of *G. nesiotis* on the cladograms (Fig. 3–5) makes sense biologically. Apparently, *G. nesiotis* became extinct on Tristan and *G. comeri* from Gough was introduced there, resulting in the current situation with *G. comeri* occurring on both islands. This implies that modern illustrations of so-called *G. nesiotis* from Tristan (Fig. 2) probably show introduced *G. comeri* from Gough.

The difference between *G. nesiotis* and *G. comeri* is most conspicuous in the D-loop sequence. This is a marker from a non-coding region, which makes it more difficult to exclude it as a potential pseudogene [16]. In some cases preferential amplification of numt (nuclear mitochondrial insertion) sequences has been observed [17,18], but in most ancient DNA studies only occasional co-amplification of numts has been reported [19,20]. This is not surprising since (particularly) in ancient DNA samples, mitochondrial DNA will be in excess over nuclear DNA. Consequently the incidence of numts should be reduced in such samples. To minimize the chance of amplifying numts, we did not use blood as a source for DNA-extractions in our recently collected material [17,21] (in the older specimens it was not possible anyway). Because ‘universal’ primers may also be particularly prone to amplification of numts [17], the primers for the D-loop were made ‘gallinule-specific’. They did not even work for the closely related coot, *Fulica atra*. Products that were sequenced directly (both strands) showed only one signal, whereas multiple signals can be expected if both the target product and a numt would have been amplified. Interclone variation was low (Dataset S1, S2, S3). Only one sequence (*G. nesiotis*, marker ATP8) out of 96 clones (Table 3) could clearly be identified as a numt (Dataset S2). No stop-codons or frame-shift mutations were observed for the coding-region datasets (ATP8 and cytochrome b). No obvious deviations in either substitution rate (pairwise relative rate test) or base composition, like a decrease in GC content [22,23], were observed.

All sequences of recently collected moorhens from Tristan were identical to those of *G. comeri* from Gough and should be considered conspecific therefore. Cross contamination is very unlikely, since specimens from Tristan and Gough were amplified in different PCR-batches and contamination was not detected in other, partly much older specimens. Most probably the sequences are identical because *G. comeri* was introduced only recently on Tristan. Genetic variation within island populations is generally small compared to mainland populations [24–26]. For example, the giant tortoises (*Aldabrachelys*) of Mahé (Seychelles) and Mauritius (Mascare islands) still have identical sequences compared to those of Aldabra, from where they were shipped since the
Figure 2. Illustrative stamps, issued in 1987 and 2005. (A) 2005: Text and illustrations belong together and are correct. (B) 1987: In Gough G. comeri occurs, not G. nesiotis; both names should not be synonymized. (C) 2005: The text correctly indicates G. nesiotis as from Tristan, but the bird itself most probably belongs to G. comeri, introduced from Gough, since G. nesiotis is now extinct on Tristan and not available to be pictured anymore.

doi:10.1371/journal.pone.0001835.g002

Table 1. Taxa and collection information.

| Species                  | Locality        | Registration number | Year | Institute          |
|--------------------------|-----------------|---------------------|------|--------------------|
| (1) G. comeri            | Tristan da Cunha| A181759             | 1993 | Pers. col. A.J. Beintema |
| (2) G. comeri            | Tristan da Cunha| A181760             | 1993 | Pers. col. A.J. Beintema |
| (3) G. comeri            | Gough Island    | Cat. no 7 ZMA 14695 | 1960 | ZMA                |
| (4) G. comeri            | Gough Island    | Cat. no 14 ZMA 14696| 1960 | ZMA                |
| (5) G. nesiotis          | Tristan da Cunha| 1864.7.30.1         | 1864 | BM                 |
| (6) G. chloropus galeata| Suriname        | RMNH 53835          | 1968 | NNM                |
| (7) G. chloropus galeata| Suriname        | RMNH 53836          | 1968 | NNM                |
| (8) G. chloropus brachyptera| S.W. Africa    | Cat. no. 21         | 1867 | NNM                |
| (9) G. chloropus brachyptera| S.W. Africa    | Cat. no. 22         | 1867 | NNM                |
| (10) G. chloropus brachyptera| Tanzania      | RMNH 43858          | 1965 | NNM                |
| (11) G. chloropus orientalis| Cheribon, Java| Cat. no 94 RMNH 26803| 1925 | NNM                |
| (12) G. chloropus indica| Chang Hwa, Taiwan| Cat. no 12 RMNH 53054| 1968 | NNM                |
| (13) G. chloropus chloropus| The Netherlands| DG2073              | 2005 | NNM                |
| (14) G. chloropus chloropus| The Netherlands| DG2077              | 2005 | NNM                |
| (15) Fulica atra         | The Netherlands | DG2071              | 2005 | NNM                |

*ZMA = zoologisch museum Amsterdam, BM = Natural History Museum, Tring, NNM = nationaal natuurhistorisch museum, Naturalis.

doi:10.1371/journal.pone.0001835.t001
1820s or earlier [27]. *G. comeri* may have been introduced on Tristan somewhere in the mid 1950s [12]. Assuming a generation time of two years, as known for *Gallinula chloropus*, *G. comeri* would only have had about only 20 generations (40 years) to differentiate on Tristan.

Both the genetic distances (Table 2) and the fact that *G. nesiotis* and *G. comeri* form a clade with the investigated moorhens of Africa/Eurasia (Fig. 3–5), suggest that the ancestor(s) of these island gallinules originated from Africa and not America, as suggested by Eber [10]. Our data do not allow us to distinguish between a single dispersal event to the archipelago, followed by allopatric differentiation, or two separate introductions from the continent to both Tristan and Gough.

As is inevitably the case with isolated island populations, the question of whether *G. nesiotis* and *G. comeri* were reproductively isolated under natural circumstances cannot be answered. Our limited data from a small number of specimens and sequences of only the mitochondrial lineage are insufficient to demonstrate hybridisation. Even though island populations generally show lower genetic variation than related mainland populations [24], the genetic distances between *G. nesiotis* and *G. comeri* are of at least the same magnitude as those found between taxa that figure as subspecies of *G. chloropus* in the literature (Fig. 5, Table 2). Therefore, we propose that the extinct moorhen of Tristan and the moorhens that live on Gough and Tristan today be regarded as subspecies, viz. *G. n. nesiotis* and *G. n. comeri*, respectively. This is in conformity with two recent, general checklists of the birds of the world [28,29] and a detailed monograph of the rails of the world [13], but is different from a morphological study by Eber [10] in which both taxa are considered synonyms.

### Materials and Methods

#### Taxa

Tissues from fourteen gallinules and a coot were put at our disposal by various institutes (Table 1). These include tissues of (I) ‘recently’ collected moorhens from Tristan da Cunha, (II) moorhens from Gough from the collection ZMA, (III) the 1864 specimen of *G. nesiotis* from the Natural History Museum, Tring, and (IV) a number of subspecies of *Gallinula chloropus* from South-America, Africa, Europe, Taiwan and Java from the National Museum of Natural History Naturalis, Leiden. The coot, *Fulica atra*, was used as outgroup.

#### DNA extraction

DNA extractions on specimens from 1968 (Table 1) and older were carried out in a dedicated aDNA facility (LAF, Leiden, the Netherlands), which is physically isolated from the main laboratories. Before extractions took place, the extraction room was cleaned with a 0.05% bleach solution and the extraction-cabinet was decontaminated by turning on the UV lights at least 1 hour prior to the start of the extractions. No more than four extractions were done at once and negative controls were included with each set of extractions. Pippetes were cleaned with bleach and subsequently decontaminated (together with the disposables) by UV irradiation (UV linker). Tissues were cut into small pieces to enlarge the contact surface between tissue and buffer. Total genomic DNA was extracted with a DNeasy Tissue Kit (Qiagen) using a prolonged incubation (24 hours). Proteinase K was added twice, once at the start and after 6 hours of incubation. To concentrate the extract, elution volume was decreased to 40 μl. Extractions on recently collected specimens (1993–2005) were done in a common lab, also using a DNeasy Tissue Kit (Qiagen).

#### PCR and sequencing

PCRs were never performed in the aDNA facility and amplicons were never stored in this building. PCRs on the extract of the 1864 specimen were duplicated in different laboratories that are physically separated from each other as well as from the aDNA facility. In none of these labs had ever been worked on any species of gallinule before. Fragments from three non-adjacent mitochondrial gene regions (679 basepairs in total; primersites excluded) were amplified by PCR: the D-loop, tRNA-Lysine/ATP8 and cytochrome b. The length of these fragments (primer sites included) was 234, 236 and 375 bp, respectively. Primer sequences and

### Table 2. Genetic distances.

| Taxa | comeri | comeri | comeri | comeri | nesiotis |
|------|--------|--------|--------|--------|----------|
| (1)  | *G. comeri* | 0/0.000 | 0/0.000 | 0/0.000 | 0/0.000 |
| (2)  | 0/0.000 | 0/0.000 | 0/0.000 | 0/0.000 | 0/0.000 |
| (3)  | 21/0.031 | 21/0.031 | 21/0.031 | 21/0.031 | 21/0.031 |
| (4)  | 40/0.059 | 40/0.059 | 40/0.059 | 40/0.059 | 41/0.061 |
| (5)  | 41/0.060 | 41/0.060 | 41/0.060 | 41/0.060 | 40/0.059 |
| (6)  | 20/0.035 | 20/0.035 | 20/0.035 | 20/0.035 | 24/0.042 |
| (7)  | 22/0.038 | 22/0.038 | 22/0.038 | 22/0.038 | 24/0.042 |
| (8)  | 22/0.032 | 22/0.032 | 22/0.032 | 22/0.032 | 25/0.037 |
| (9)  | 19/0.033 | 19/0.033 | 19/0.033 | 19/0.033 | 21/0.037 |
| (10) | 42/0.070 | 42/0.070 | 42/0.070 | 42/0.070 | 25/0.037 |
| (11) | 44/0.076 | 42/0.073 | 6/0.010 | 6/0.010 | 6/0.010 |
| (12) | 43/0.063 | 42/0.062 | 6/0.000 | 6/0.000 | 6/0.000 |
| (13) | 40/0.070 | 40/0.070 | 17/0.030 | 15/0.026 | 17/0.030 |
| (14) | 46/0.068 | 45/0.066 | 5/0.009 | 5/0.009 | 5/0.009 |
| (15) | 44/0.065 | 43/0.063 | 4/0.007 | 4/0.007 | 4/0.007 |
| (16) | 93/0.138 | 94/0.139 | 93/0.163 | 91/0.160 | 103/0.152 |
| (17) | 18/0.031 | 18/0.031 | 18/0.031 | 18/0.031 | 18/0.031 |
| (18) | 16/0.028 | 16/0.028 | 16/0.028 | 16/0.028 | 16/0.028 |
| (19) | 15/0.026 | 15/0.026 | 15/0.026 | 15/0.026 | 15/0.026 |
| (20) | 94/0.166 | 105/0.155 | 104/0.154 | 103/0.152 | 103/0.152 |
references are described in Table 4. For *G. nesiotes* and (most) specimens of 1960 and older, cytochrome *b* could not be amplified directly using primers L14841 and H15149 [30]. Presumably because the DNA within these specimens got too degraded over time. Therefore, internal primers were designed (Table 4) to amplify this fragment in two overlapping parts: L14841-Rev219 (219 bp) and Fwd141-H15149 (249 bp). The primers for the D-loop (CR-OUD-F and CR-OUD-R) are ‘gallinule-specific’. For the coot, *Fulica atra*, the same region had to be amplified with other primers: CR-175-F and 12S-29-R (Table 4). PCRs were done using a standard Taq DNA Polymerase kit (Qiagen). Reaction volume was 25 μl and PCR conditions were 0.4 μM of each primer, 0.2 mM dNTP’s and 5 units of Taq DNA Polymerase. For amplification of the cytochrome *b* and ATP8 regions, the final concentration of MgCl₂ was 2.5 mM. For amplification of the D-loop fragment no MgCl₂ was added (1.5 mM was already in the Qiagen PCR-buffer). Thermo-cycling conditions were 3 min. at 94°C (initial denaturation), followed by 40 cycles (15 sec. at 94°C, 30 sec. at AT°C and 40 sec. at 72°C) and final extension 5 min. at 72°C. Where AT is the annealling temperature for each primerset; 50°C for both cytochrome *b* and ATP8, 55°C for the D-loop fragment and 57°C for reamplification of cloned products (see below).

All PCR products from *G. nesiotes* and a number of PCR product from the D-loop of selected taxa (Table 3) were cloned using either pGEM®-T Easy Vector System from Promega or Topo TA Cloning® from Invitrogen. At least three colonies were picked per plate and used to initiate reamplifications with primers 21M13_F and 21M13_R (Table 4). Reamplified products were cleaned using a Nucleospin® kit (Macherey-Nagel). Subsequently these products were sequenced either in-house on a Megabace™ 1000 DNA Analysis System (Amersham), or on a 3730xl DNA analyzer (Applied Biosystems) at Macrogen Inc. (Korea) using only primer 21M13_F. All other PCR products were cleaned (same procedure) and sequenced directly (both directions) with their respective PCR primers (Table 4). A summary of the specimens and the number of colonies sequenced per target region is given in Table 3. Sequences were assembled using Sequencher version 4.2 (Gene Codes Corporation) and aligned manually using MacClade version 4.08 [31]. The sequences were deposited in GenBank (accession numbers EF681971-EF682015).

Phylogenetic analysis

For phylogenetic analyses, all sequences (all regions; consensus sequences when products were cloned) were put in a single datamatrix (Dataset S4; an ILD-test showed no incongruence between the regions, p = 0.971) and *Fulica atra* was designated as outgroup. To get branch support values, we performed phylogenetic analyses with three methods: Neighbour-Joining (PAUP ver. 4.0b2a [32]), Maximum Likelihood (PAUP ver. 4.0b2a [32]) and Bayesian analysis (MrBayes ver. 3.1.2 [33]). For the NJ analysis, we performed a bootstrap analysis (1000 replicates, optimality criterion set to distance) and calculated a 50% majority rule consensus cladogram (Fig. 3). For the ML analysis, the HKY+G model was selected by Modeltest ver. 3.7 [34] with the following parameters: Tratio = 10.660, gamma shape parameter = 0.0941, base frequencies A = 0.3473, C = 0.3004, G = 0.1341, T = 0.2182.
and proportion of invariable sites (pinvar) = 0. A bootstrap analysis (1000 replicates, 5 random additions per bootstrap replicate and TBR branch swapping) was performed and a 50% majority rule consensus tree was calculated (Fig. 4). For the MrBayes analysis, the best-fit model for each partition (four partitions: tRNA-Lysine and ATP8 were considered as two partitions) was selected by hLRT in MrModeltest ver. 2.2 [35]: D-loop (HKY+Y), tRNA-Lysine (HKY), ATP8 (GTR+I) and cytochrome b (GTR+I). A dirichlet (1,1,1,1) prior was specified on the state frequencies for all partitions, except for the tRNA-Lysine partition, where the frequencies were equal. All partitions had different rates for transition and transversions (nst = 2), except for tRNA-Lysine (nst = 1). Among-site rate variation was equal for tRNA-Lysine, gamma-distributed for both D-loop and ATP8 and for cytochrome b a proportion of the sites was invariant. Two runs (set up for 10 000 000 generations) were performed simultaneously (4 chains per run) in MrBayes ver. 3.1.2 [34] and the convergence diagnostic was set to 0.009. A Markov chain Monte Carlo (MCMC) analysis was done with swapfreq = 2, temp = 0.002 and samplefreq = 100; convergence was reached after 165 000 generations. The trees of both runs (3302 in total) were combined (2702: burnin was set to 300) and a 50% majority rule consensus tree (contype = halfcom-pat) was calculated (Fig. 5).

Genetic distances

Genetic distances (absolute number of changes and uncorrected “p” distances) were calculated with Paup ver. 4.0b2a [32] based on the combined dataset (Dataset S4).

Pairwise relative rates test

With Fulica atra specified as outgroup, a Pairwise Relative Rate Test [36] as implemented in HyPhy [37] using the HKY model (as specified by Modeltest ver. 3.7 [34]) was performed on the combined dataset (Dataset S4).

Supporting Information

Dataset S1 Cloning results of G. nesiotis for ATP8. Found at: doi:10.1371/journal.pone.0001835.s001 (0.00 MB TXT)

Dataset S2 Cloning results of G. nesiotis for D-loop. Found at: doi:10.1371/journal.pone.0001835.s002 (0.00 MB TXT)

Dataset S3 Cloning results of G. nesiotis for cytochrome b. Found at: doi:10.1371/journal.pone.0001835.s003 (0.01 MB TXT)

Dataset S4 Combined dataset showing all taxa and all markers (D-loop, ATP8 and cytochrome b) used in this study. Found at: doi:10.1371/journal.pone.0001835.s004 (0.01 MB TXT)

Acknowledgments

We thank H. van Grouw (National Museum of Natural History Naturalis, Leiden, The Netherlands), Dr R. Pryce-Jones (The Natural History Museum, Tring, U.K.) and T. Prins (Zoological Museum, University of Amsterdam, the Netherlands) who provided most valuable material for
DNA analyses. We also thank H. Cross (National Herbarium the Netherlands, Leiden) for technical assistance, K. Vrieling (Leiden University, the Netherlands) for putting at our disposal the Topo TA Cloning® System (Invitrogen) and P. Brakefield (Leiden University, the Netherlands) for reviewing our manuscript. Finally we acknowledge the Tristan da Cunha Post Office, who gave permission to illustrate this manuscript with some of their stamps (Fig. 1) and we acknowledge the Crown Agents Stamp Bureau for facilitating correspondence.

Figure 5. Bayes 50% majority rule consensus ('halfcompat') phylogram (branch lengths are proportional to the expected number of substitutions). Values indicate branch support by Bayesian inference.
doi:10.1371/journal.pone.0001835.g005

Table 3. Number of colonies sequenced per target region per taxon.

| Taxon                        | Year | D-loop | tRNA-Lysine/Atp8 | cytochrome b |
|------------------------------|------|--------|------------------|--------------|
| (1) G. comeri                | 1993 | -      | -                | -            |
| (2) G. comeri                | 1993 | -      | -                | -            |
| (3) G. comeri                | 1960 | 4      | -                | -            |
| (4) G. comeri                | 1960 | -      | -                | -            |
| (5) G. nesiotis              | 1864 | 8 (5/3)* | 14 (7/7)*       | 23 (6/5-6/6)*|
| (6) G. c. galeata            | 1968 | 3      | -                | -            |
| (7) G. c. galeata            | 1968 | 4      | -                | -            |
| (8) G. c. brachyptera        | 1867 | -      | -                | -            |
| (9) G. c. brachyptera        | 1867 | 5      | -                | -            |
| (10) G. c. brachyptera       | 1965 | 3      | -                | -            |
| (11) G. c. orientalis        | 1925 | 5      | 4                | 7 (7-8)*     |
| (12) G. c. indica            | 1968 | 4      | 5                | 7 (5-2)*     |
| (13) G. c. chloropus         | 2005 | -      | -                | -            |
| (14) G. c. chloropus         | 2005 | -      | -                | -            |
| (15) F. atra                 | 2005 | -      | -                | -            |

*Within parentheses are the number of colonies sequenced for each PCR product.
doi:10.1371/journal.pone.0001835.t003
Table 4. PCR and sequencing primers.

| Primer name | Primer sequence (5’ to 3’) | Target | Reference |
|-------------|-----------------------------|--------|-----------|
| L14841      | AAAAGGCTTCCATCCACACATCTGATGTTAAA | cytochrome b | Kocher, 1989 |
| H15149      | AAAACGAGCGCCCTCGAGTAAGTATTTTGCCTCA | cytochrome b | Kocher, 1989 |
| Fwd141      | CCAACATGCGGACAGTACAATA | cytochrome b | This study |
| Rev219      | GCATGATGAGAAGAATGAGCCCTCC | cytochrome b | This study |
| L9051       | CAGCCTAGGCTTTTAAG | tRNA-Lys/ATP8 | Slikas, 2002 |
| H9241       | TGGTGGAGGCTGATGTTCA | tRNA-Lys/ATP8 | Slikas, 2002 |
| CR-OUd-F    | CAAGGATTAAATGATATAGCCTTACC | D-loop | This study |
| CR-OUd-R    | TGATACATTGTGTGTTGATGAA | D-loop | This study |
| CR-175-F    | GAGGATACTTATGTTGACCTGAG | D-loop | This study |
| 12S-29-R    | TTAAAACTGGGGAAGGACTGTCA | D-loop | This study |
| 21M13_F     | TGGTAAAAAGCCCAGGCAATG | PCR 2.1-TOPO M13 priming site | TOPO TA Cloning kit |
| 21M13_R     | CAGGAAAAGCGTATGAC | PCR 2.1-TOPO M13 priming site | TOPO TA Cloning kit |

Author Contributions
Conceived and designed the experiments: DG. Performed the experiments: DG. Analyzed the data: DG. Wrote the paper: DG. Other: Provided a hypothesis and some of the specimens: AB. Reviewed the manuscript: EG RD AB.

References
1. Sclater PL (1861) On the Island-Hen of Tristan d’Acunha. Proceedings of the Zoological Society London. pp. 260–263.
2. Britenma AJ (1972) The history of the Island Hen (Gallinula nesiotis), the extinct flightless gallinule of Tristan da Cunha. Bulletin of the British Ornithologists’ Club 92: 106–112.
3. Allen JA (1892) Description of a new Gallinule from Gough Island. Bulletin of the American Museum of Natural History 4: 57–58.
4. Milner J, Brierly OW (1869) The Cruise of H.M.S. ‘Glatea’, Captain H.R.H. the Duke of Edinburgh, KG., in, 1867–1868. London: W.H. Allen.
5. Sperling RM (1872) Letter on Tristan da Cunha. Ibis 3: 74–79.
6. Nicoll MJ (1906) On the Birds collected and observed during the Voyage of the ‘Valhalla’. 8: 666–712.
7. Nicoll MJ (1980) Three Voyages of a Naturalist; being an Account to Many Little-Known Islands in the Three Oceans visited by the ‘Valhalla’. London: R.Y.S. Witherby & Co.
8. Knox AG, Waythes MF (1994) Extinct and endangered birds in the collections of the Natural History Museum. British Ornithologist’s Club Occasional Publications 1.
9. Broekhuyzen GJ, Macnae B (1949) Observations on the birds of Tristan da Cunha Islands and Gough Island in February and early March, 1948. Ardea 37: 97–113.
10. Ember G (1961) Vergleichende untersuchungen am flugfahigen Teichhuhn Gallinula chl. chloropus und an der flugunfa¨higen Inselralle Gallinula nesiotis. Bonner Zoologische Beitrage 12: 247–315.
11. Slikas B, Olson SL, Fleischer RC (2002) Rapid, independent evolution of flightlessness in four species of Pacific Island rails (Rallidae): an analysis based on mitochondrial chollox sequence data. Journal of Avian Biology 33: 5–14.
12. Richardson ME (1984) Aspects of the ornithology of the Tristan da Cunha group and Gough Island, 1972-1974. Cormorant 12: 123–201.
13. Taylor B, van Perlo B (1998) Rails. A guide to the Rails, Coots, Crakes and Gallinules of the world. Sussex: Pica Press.
14. Binladen J, Winf C, Gilbert MTP, Bunce M, Barnett R, et al. (February 2006) Assessing the fidelity of ancient DNA sequences amplified from nuclear genes. Genetics: 172: 733–741.
15. Gilbert MTP, Willerslev E, Hansen AJ, Barnes I, Ruddle B, et al. (January 2003) Distribution patterns of postmortem damage in human mitochondrial DNA. Am J Hum Genet 72: 32–47.
16. Perna NT, Kocher TD (1996) Mitochondrial DNA: molecular fossils in the nucleus.Curr Biol 6: 128–129.
17. Snellen JA, Quinteros J, Quinteros J, Quinteros J, Quinteros J, et al. (January 2003) Mitochondrial DNA evolution in Animals- Amplification and Sequencing with Conserved Primers. Proc. Natl. Acad. Sci. U. S. A. 100: 6196–6200.
18. Maddison WP, Maddison DR (2000) MacClade: analysis of phylogeny and character evolution, version 4.0. Sunderland, Sinauer.
19. Swofford DL (1998) PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods), Version 4. Sunderland, MA: Sinauer Associates.
20. Ronquist F, Huelsenbeck JP (2003) MrBayes: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. doi:10.1093/bioinformatics/19.19.1572.
21. Swoford DL (1998) PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods), Version 4. Sunderland, MA: Sinauer Associates.
22. Ronquist F, Huelsenbeck JP (August 12, 2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. doi:10.1093/bioinformatics/19.19.1572.
23. Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817–818. doi:10.1093/bioinformatics/14.9.817.
24. Nakamura JAA (2004) MrModeltest 2.2. Program distributed by the author. Uppsala University: Evolutionary Biology Centre.
25. Muse SV, Weir BS (1992) Testing for Equality of Evolutionary Rates. Genetics 132: 269–276.
26. Pond SLK, Frost SDW, Muse SV (2005) HyPhy: hypothesis testing using phylogenies. Oxford Univ Press. pp 676–679.