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Divergence between passerine populations from the Malvinas – Falkland Islands and their continental counterparts: a comparative phylogeographical study

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Bursts of speciation have followed colonization of remote oceanic islands by diverse taxa, a process evidenced by island endemics around the world. The present study explores whether the Malvinas – Falkland Islands (MFI), a relatively understudied archipelago off the South Atlantic coast of Patagonia, harbour endemic genetic lineages of passerine birds. Nine passerine species nest regularly in the MFI (*Cinclodes antarcticus*, *Muscisaxicola maclovianus*, *Troglodytes cobbi*, *Cistothorus platensis*, *Turdus falcklandii*, *Anthus correndera*, *Melanodera melanodera*, *Sturnella loyca*, and *Carduelis barbata*). Mitochondrial DNA (cytochrome *c* oxidase I sequences) are used to quantify and compare divergence between insular and continental populations, finding genetic patterns to vary across these nine species. Most MFI passerines do not show significant genetic differentiation from continental populations, whereas *C. platensis*, *M. melanodera*, and *T. falcklandii* are modestly diverged. Finally, *T. cobbi* differes markedly from its closest continental relative *Troglodytes aedon*, a result that is confirmed using nuclear and vocal data. The study also identifies broadly divergent lineages within continental populations of *C. platensis* and *T. aedon*. Taken together, these results suggest that the land bird populations of the MFI were established at different times. *Troglodytes cobbi* is the oldest MFI land bird, splitting from continental *T. aedon* during the Great Patagonian Glaciation of the Pleistocene. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **106**, 865–879.

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Island avifaunas around the world have inspired the development of many central ideas in evolutionary biology. Mockingbird diversity of the Galápagos Islands was crucial to Darwin's conception of speciation by natural selection (Darwin, 1839), birds from the Solomon islands helped shape Mayr's view of allopatric speciation (Mayr, 1940), and examples of diversification *in situ* such as Darwin's Finches and drepanidine Hawaiian Honeycreepers have provided key insights on adaptive radiations and ecological speciation (Price, 2007). The colonization of islands through rare dispersal events leads to rapid reproductive isolation of small groups of individuals, following which genetic drift can play an important role during divergence in allopatry if populations remain small (Vincek *et al.*, 1997). Islands may also impose

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different selective pressures in relation to the adjacent continent; differing in climate, overall species richness and population densities, risks of predation, dispersal costs and other ecological variables (MacArthur & Wilson, 1967). Given these evolutionary drivers, it is not unexpected that a significant portion of the extant global avian fauna has evolved in association with insularity, with approximately 17% of all bird species confined to archipelagos (Johnson & Stattersfield, 1990). Thus, island avifaunas provide important study systems for diversification and speciation.

The Malvinas – Falkland Islands (MFI) comprise the largest archipelago in the South Atlantic, located approximately 450 km north-east of the coast of Tierra del Fuego, the southern tip of South America. The MFI archipelago consists of two main and approximately 750 smaller offshore islands, totalling approximately 12 000 km^2 in area (Woods, 2001). Sea level fluctuations during the various Pleistocene glacial cycles caused the distance between the continent and the islands to vary. During the last glacial maximum (LGM: approximately 24 000 years BP), the sea level was 120 to 140 m lower, causing the MFI to form a single large landmass approximately 220 km from the continent (Ponce *et al*., 2011). Moreover, connectivity with the continent might have been accentuated by the existence of a now-submerged island to the south, which may have provided a stepping-stone between Tierra del Fuego and the MFI (Ponce *et al*., 2011). The existence of a land bridge with the continent during the Pleistocene but before the LGM has also been proposed (Ponce *et al*., 2011). As a consequence of its proximity to (and possible connectivity with) the continent, the biota of the MFI is derived from that of Patagonia, and particularly Tierra del Fuego (McDowall, 2005; Morrone & Posadas, 2005). The MFI possess endemic species, including several vascular plants (Woods, 2000; McDowall, 2005), numerous invertebrates (McDowall, 2005; Papadopoulou *et al*., 2009), the Falkland Steamer Duck (or Pato Vapor Malvinero, *Tachyeres brachypterus*, Latham 1790) (Woods & Woods, 1997, 2006), and the recently extinct Falkland Islands Wolf (or Zorro Malvinero, *Dusicyon australis*, Kerr 1792), which attracted Darwin's attention during his visit to the archipelago (Darwin, 1838; Slater *et al*., 2009).

Various remote islands of the South Atlantic harbor endemic land bird species. *Anthus antarcticus* (Cabanis 1884) inhabits the approximately 4000 km^2 island of South Georgia, *Rowettia goughensis* (Clarke 1904) is endemic to the 65 km^2 Gough Island, and two species of *Neospiza* buntings (Salvadori 1903) have radiated *in situ* in the approximately 200 km^2 Tristan da Cunha archipelago (Ryan *et al*., 2007). The MFI comprise the largest archipelago in the region, although they have no recognized endemic land bird species yet (Remsen *et al*., 2011), perhaps because their proximity with the continent allows elevated levels of gene flow that prevent divergence. The recorded avifauna of the MFI consists of over 200 bird species (Woods & Woods, 1997, 2006), which includes many passerines that also occur in Patagonia, although the majority are vagrants that do not breed in the islands (Woods & Woods, 1997; Remsen *et al*., 2011). Only nine passerine taxa breed regularly in the MFI: *Cinclodes antarcticus antarcticus* (Garnot 1826), *Muscisaxicola maclovianus maclovianus* (Garnot 1829), *Troglodytes cobbi* Chubb 1909, *Cistothorus platensis falklandicus* Chapman 1934, *Turdus falcklandii falcklandii* Quoy & Gaimard 1824, *Anthus correndera grayi* Bonaparte 1850, *Melanodera melanodera melanodera* (Quoy & Gaimard 1824), *Sturnella loyca falklandica* (Leverkuhn 1889), and *Carduelis barbata* (Molina 1782) (Woods & Woods, 1997, 2006). All but *Carduelis barbata* are taxa found exclusively in the MFI and, with the exception of perhaps *Troglodytes cobbi*, do not show marked morphological differences with respect to their respective continental populations (Woods & Woods, 1997, 2006). *Troglodytes cobbi* is currently considered conspecific with *Troglodytes aedon* (Vieillot 1809) (Remsen *et al*., 2011) but has been suggested to merit full species status on the basis of differences in morphology (shape and overall size), plumage, behaviour, and ecology (Woods, 1993; Kroodsma & Brewer, 2005).

To our knowledge, only one study has compared the phylogeographical pattern between continental and MFI populations of a bird species. McCracken & Wilson (2011) found significant differentiation between continental and insular populations of speckled teal (*Anas flavirostris*, Vieillot 1816) despite significant gene flow from the continent to the islands, suggesting divergent lineages of less mobile land bird species could also exist in the MFI. The present study is the first to use DNA sequences to compare the passerine populations of the MFI to their continental counterparts. We explored whether the MFI have contributed to the isolation of these bird populations, testing if the designated endemic putative species (*T. cobbi*) and eight other passerine taxa (see above) represent distinct genetic lineages. Specifically, we used DNA sequence data to: (1) quantify divergence between continental and insular populations of all the MFI passerine species; (2) compare phylogeographical patterns across species to gain insight into the colonization history of the islands; and (3) test whether *T. cobbi* is likely to constitute a full MFI-endemic species, combining both DNA and song evidence.

MATERIAL AND METHODS

Taxon sampling and datasets

Samples used for genetic analyses were either collected by one of the authors (J.S.C.) or obtained during field trips organized by the Ornithology Division of the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN). Some samples were also taken from museum study skins housed at the MACN. All nine species of passerines that reproduce regularly in the MFI (Woods & Woods, 1997) were included in the present study, ranging from one to 69 continental samples and from one to 11 insular samples per species. For continental populations, we included an average of seven localities (range = 1–30), whereas the majority of insular samples were obtained from Sea Lion Island. For most continental samples, a voucher consisting of a study skin, skeleton or specimen in ethanol is deposited either at the MACN or at another institution. For blood samples, species were unambiguously identified in the field and therefore also included in the present study. Details for the samples used in this study are provided in the Supporting information (see Supporting information, Table S1). Sampling localities (Fig. 1) were grouped by geographical proximity or with respect to major geographical barriers (e.g. the Andes Mountains) to display results clearly. From North to South, samples were assigned to eight groups: Bolivia (BOL); Jujuy, Argentina (JUJ); Northeastern Argentina (NEA); Central Argentina (CEN); Chile (CHI); Central Patagonia, Argentina (CPA); Tierra del Fuego, Argentina (TDF); and Malvinas – Falkland Islands (MFI). For *C. platensis* and *T. aedon*, phylogeographical structure was found within continental populations (see Results), and even within particular clusters of localities. In these cases, different genetic lineages were observed coexisting within the same or neighbouring localities, suggesting that by clustering localities for simplicity in displaying our results, we were not masking the limits between lineages.

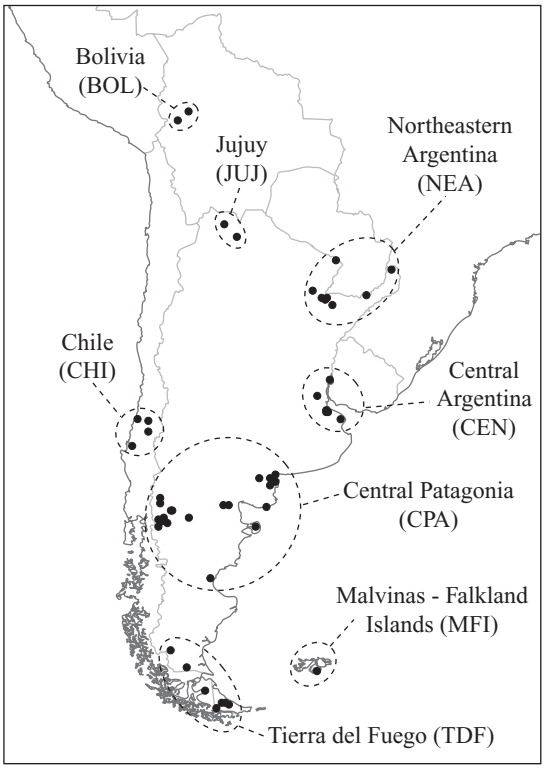

Figure 1. Localities where at least one of the nine surveyed species was sampled. For simplicity, sites were grouped by geographical proximity or with respect to geographical barriers.

We chose cytochrome *c* oxidase I (COI) to initially assess intraspecific divergence because this locus is used in DNA barcoding with high success in separating and identifying sister or cryptic avian species (Hebert *et al*., 2003; Kerr *et al*., 2009). Moreover, using this marker various studies have identified phylogeographical structure within Neotropical bird species (Kerr *et al*., 2009; Campagna *et al*., 2011). Some COI sequences were downloaded from BOLD (http://www.boldsystems.org) and had been obtained previously as part of an ongoing project from our group to barcode the birds of Argentina (Kerr *et al*., 2009). We did not have access to continental samples from *C. antarcticus*; however, one previously published COII sequence (Chesser, 2004) was available on GenBank (http://www.ncbi.nlm.nih.gov). Thus, for this species, we used COII to assess intraspecific divergence.

For taxa showing divergent lineages between continental and insular populations, we selected a subset of samples to verify the genetic divergence using additional mitochondrial and nuclear genes. For these samples, we added sequences from cytochrome *b* (cyt *b*); one Z-linked marker, chromodomain-helicase-DNA binding protein (CHD1); and one autosomal marker, intron 5 of the β-fibrinogen gene (FGB).

DNA extraction, amplification, and sequencing

DNA sources for use in the present study included frozen pectoral muscle, blood conserved in ethanol or lysis buffer and toe pads or feathers from museum study skins. DNA extraction was conducted as in Campagna *et al*. (2012). We amplified a total of five gene fragments using polymerase chain reaction (PCR). Amplification of 694 COI base pairs (bp) was conducted as in Kerr *et al*. (2009), COII (approximately 600 bp) PCRs followed (Chesser, 1999, 2000), cyt *b* PCRs (approximately 920 bp) followed conditions outlined by Sato *et al*. (2001), FGB PCR

conditions (approximately 560 bp) are summarized in Campagna *et al*. (2011), and CHD1 PCRs (approx. 620 bp) are described in Campagna *et al*. (2012). The supporting information (see Supporting information, Table S2) shows the primer sequences with their original references, annealing temperatures and $MgCl_2$ concentrations used for all PCR reactions in the present study. PCR products were visualized on a 2% agarose gel using ethidium bromide, purified using the QIAquick PCR purification Kit (Qiagen) in accordance with the manufacturer's instructions, and sequenced bi-directionally with the primers used for amplification. Sequencing was conducted at the Canadian Centre for DNA Barcoding (Guelph, ON, Canada) or the London Regional Genomics Centre (London, ON, Canada). Sequences were deposited in GenBank. Accession numbers are provided in the Supporting information (see Supporting information, Table S1).

Genetic analysis

Sequences were aligned using BIOEDIT, version 7.0.9.0 (Hall, 1999), and protein-coding sequences were visually inspected to verify lack of indels and then translated into amino acids to confirm absence of stop codons. The phylogeographical structure within each species was initially explored by constructing a statistical parsimony network (Templeton, Crandall & Sing, 1992) using TCS, version 1.21 (Clement, Posada & Crandall, 2000) to represent the genealogical relationships among the COI haplotypes found (COII for *C. antarcticus*). Additionally, the degree of divergence between continental and insular populations was assessed using two metrics of genetic divergence: Kimura two-parameter (K2P) distances (Kimura, 1980) and *F*-statistics (the F_{ST} analogue – Φ_{ST}). K2P estimates evolutionary distances in terms of the number of nucleotide substitutions, giving different weight to transitions and transversions. This metric is used for species-level analyses in DNA barcoding (Hebert *et al*., 2003), where there are typically few substitutions among sequences (Nei & Kumar, 2000). COI K2P distances were calculated using MEGA, version 4 (Tamura *et al*., 2007). Φ_{ST} measures the proportion of total genetic variation explained by differences between populations, accounting for the degree of divergence between haplotypes by constructing a pairwise K2P distance matrix. Φ_{ST} calculations were completed using ARLEQUIN, version 3.5.1.2 (Excoffier & Lischer, 2010) and significance was tested through 1000 random permutations with sequential Bonferroni corrections (Rice, 1989).

For *T. aedon* and *T. cobbi*, our TCS analysis could not confidently connect all COI haplotypes and identified five independent networks. To explore the affinities among these networks we performed a Bayesian phylogenetic analysis using MrBayes, version 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). To root the tree we used sequences available on GenBank from closely related species (*T. solstitialis*, Sclater 1859; *T. troglodytes*, Linnaeus 1758; *C. platensis*; and *C. palustris*, Wilson 1810). We selected the model of nucleotide evolution for the COI dataset with JMODELTEST, version 0.1.1 (Posada, 2008), carrying out likelihood calculations using 11 substitution schemes, unequal base frequencies, a proportion of invariable sites, rate variation among sites with four rate categories and a fixed tree estimated with the BIONJ option. The model that best fit the data according to the Akaike information criterion was the TrN (Tamura & Nei, 1993) with gamma-distributed rate variation across sites (+G) and a proportion of invariable sites (+I). Using the closest available model to this, we ran two independent Bayesian analyses with four chains under default priors for all parameters for six million generations in MrBayes, until the standard deviation of split frequencies was < 0.01, indicating convergence. We sampled trees every 100 generations, and discarded the first 25% as burn-in. The 'cumulative' and 'compare' functions implemented in the software AWTY (Wilgenbusch, Warren & Swofford, 2004) were used to confirm that runs had reached stationarity. The potential scale reduction factor (Gelman & Rubin, 1992) was also very close to one for all parameters, indicating that we had adequately sampled the posterior distributions. Finally, a 50% majority rule consensus was obtained from the combined posterior tree distribution of both runs.

Divergence time between continental and insular populations was estimated using time to most recent common ancestor (TMRCA) with the Bayesian approach implemented in the BEAUti/BEAST, version 1.4.8 package (Drummond & Rambaut, 2007). We used the cyt *b* data and a calibration of 2.1% per million years (Weir & Schluter, 2008). The analysis was run for 100 million generations using a GTR + I + G model of nucleotide substitution with four rate categories, assuming a constant population size and a relaxed uncorrelated lognormal clock. Convergence in parameter estimates was checked by verifying that trends were not observed in traces of parameters and that effective sample sizes were adequate using TRACER, version 1.4 (Rambaut & Drummond, 2007).

The nuclear sequences obtained (CHD1 and FGB) showed populations-specific diagnostic sites in some cases and/or segregating sites in others. Thus, K2P distances and Φ_{ST} calculations were undertaken as described above for both the obtained sequences and inferred haplotypes. Haplotypes for each locus were inferred using DNASP, version 5.10 (Librado & Rozas,

2009) and used in subsequent analyses only if they had sites with assignment probabilities ≥ 0.95. When a combination of the number of segregating sites and sample sizes made recovery of useful haplotypes low, we divided each gene in half and inferred the haplotypes separately for both the 5′ and 3′ portions of each locus. In these cases we calculated Φ_{ST} and genetic distances for the 5′ and 3′ fraction of each locus separately.

For those species showing evidence of divergence between continental and insular populations, estimations of effective population sizes, splitting times and bi-directional migration were obtained with IMa2 (Hey & Nielsen, 2007; Hey, 2010) using data from COI and CHD1 and FGB inferred haplotypes. Sequences from both nuclear loci tested negative ($P > 0.05$) for recombination using the Phi test (Bruen, Philippe & Bryant, 2006) implemented in SPLITSTREE, version 4 (Huson & Bryant, 2006). For those cases in which we inferred haplotypes for the 5′ and 3′ portions of each locus separately, runs were conducted independently using COI data and both possible random combinations of CHD1 and FGB 5′ or 3′ fragments. Because similar results were obtained for both sets, we randomly chose one for subsequent runs. We used the infinite site mutation model (Kimura, 1969) for those loci that had only one base segregating in the sample. When more than one base was segregating in the sample at a certain position, the Hasegawa, Kishino and Yano model (Hasegawa, Kishino & Yano, 1985) was used. M mode runs showed adequate mixing with 100 chains, the geometric heating model and a burn-in period of 300 000 generations. Once we had completed preliminary analyses to determine adequate priors for each parameter, we ran IMa2 three or four times per species with different random seeds until at least 120 000 genealogies were saved. Finally, joint-posterior density estimations of model parameters were obtained in L mode. Estimations of population migration rates in both directions ($2Nm$), the effective number of migrants per generation, were obtained from the migration (m_{12}/μ or m_{21}/μ, where μ is the mutation rate) and θ ($4N_1\mu$ or $4N_2\mu$) parameters calculated with IMa2. By calculating $2Nm = 4N\mu \times 1/2 \times m/\mu$, an estimation of $2Nm$ was obtained independently of the mutation rate (Hey & Nielsen, 2004).

Song variation in *T. aedon* and *T. cobbi*

The degree of genetic isolation found between *T. aedon* and *T. cobbi* populations prompted us to search for possible differences in song, a key component of bird mate recognition systems (Price, 2007), between males of these two putative species. Song recordings from adult males were obtained by the authors (R.W.W. and S.I.) and from commercially available recordings (see Supporting information, Table S3). In total, our dataset included vocalizations from 21 *T. aedon* and 16 *T. cobbi* individuals. Recordings from 16 different continental and two insular localities were included for *T. aedon* and *T. cobbi*, respectively. Seven of the 16 continental localities occur in Patagonia, the most geographically proximate part of South America to the MFI.

Songs were analyzed with RAVEN PRO, version 1.4 (http://www.birds.cornell.edu/raven) using default settings and assessing variables *sensu* Toews & Irwin (2008) to recognize cryptic species within populations of winter wrens (*Troglodytes troglodytes*). Briefly, the seven variables measured were: *Length*, song length (s); *Min*, minimum frequency of the song (kHz); *Max*, maximum frequency of the song (kHz); *Mean*, mean frequency of the song (kHz) obtained by sampling the sound with the largest amplitude every 0.25 s (silences between notes were discarded); *SD freq*, the standard deviation in frequency (kHz) across the time points used to determine *Mean*; *Percent blank*, the number of points used to calculate *Mean* where the bird was not singing divided by the total number of points; *Trans sec*$^{-1}$, the number of times per second in which the fundamental frequency of sound changes from below to above (or *vice versa*) the mid-point in frequency range of the song (3.5 kHz). When more than one song was available for a given individual (average = 6.4, range = 2–19), we obtained mean values across all songs for the seven variables. All variables except *Percent blank* were log-transformed prior to analyses.

In our genetic analyses, the closest relatives of *T. cobbi* were identified as a lineage within *T. aedon* that included all Patagonian individuals (see Results). Thus, we compared *T. cobbi* songs not only with all continental *T. aedon* songs pooled, but also exclusively with those obtained from Patagonia. Differences in each of the seven variables were assessed separately using *t*-tests. We also employed a discriminant function analysis (DFA) entering variables simultaneously using SPSS, version 15.0 (SPSS Inc.) to assess differences between the two pre-defined groups. The ability to predict group membership for each individual was compared with the actual group membership through a jackknifed classification procedure (Tabachnick & Fidell, 2001).

RESULTS

Genetic divergence between insular and continental passerine populations

The nine passerine species analyzed show different genetic patterns when continental populations are compared with those of the MFI: Six species share

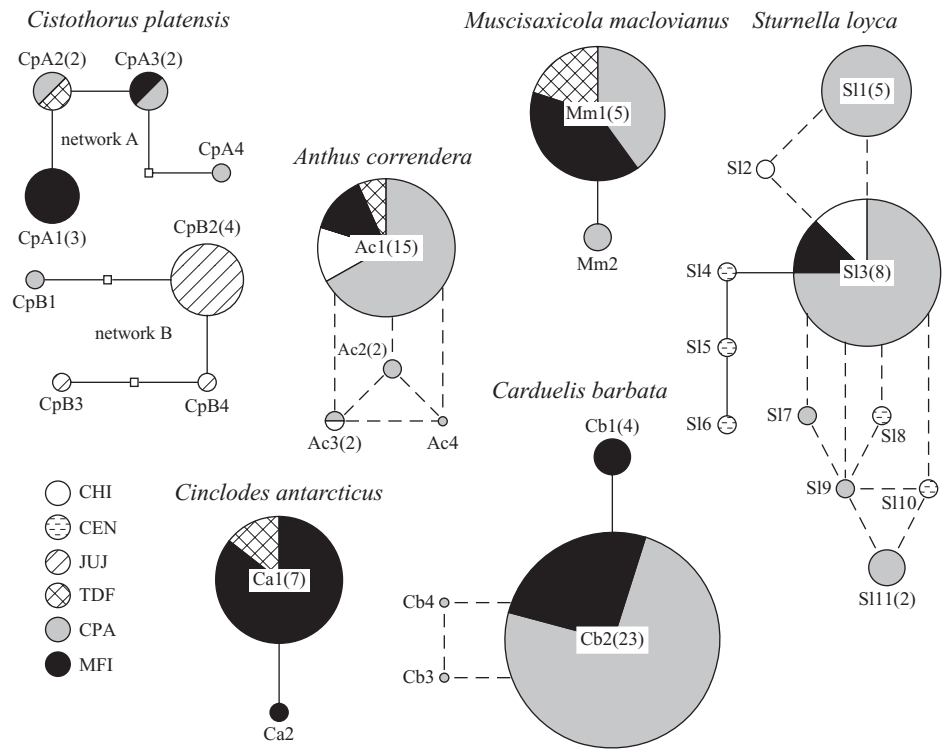

Figure 2. Unrooted maximum parsimony network showing 95% probability linkages among cytochrome *c* oxidase I (COI) haplotypes obtained from *Muscisaxicola maclovianus*, *Cistothorus platensis*, *Anthus correndera*, *Sturnella loyca*, and *Carduelis barbata*; and COII haplotypes from *Cinclodes antarcticus*. Solid lines represent single mutational changes and dashed lines show alternative connections that were not unambiguously resolved by the analysis. Empty squares represent unsampled or extinct haplotypes. Haplotypes are numbered and named with the first letters of the species where they were found and the number of individuals carrying them (when more than one) is indicated in parenthesis. Note that the TCS analysis could not confidently link all *C. platensis* haplotypes in the same network and thus assigned them to two different networks (A and B). The areas of circles are proportional, within each network, to the number of individuals possessing that haplotype. The locality where each haplotype was found is coded in accordance with Fig. 1.

COI (or COII for *C. antarcticus*) haplotypes between the two regions (Fig. 2); *M. melanodera* and *T. falcklandii* insular populations have private haplotypes differing by one mutational step from continental ones (Fig. 3); and, finally, *T. cobbi* has a single, unique haplotype separated by at least ten mutational steps from those of *T. aedon* (Fig. 4). Φ_{ST} and K2P genetic distances calculated to evaluate divergence in COI data for those species with larger sample sizes ranged from 0.00 (*C. barbata*) to 0.89 (*T. aedon/T. cobbi*) and 0.15% (*M. melanodera*) to 2.17% (*T. aedon/T. cobbi*), respectively. We observe the most striking differentiation between *T. aedon* and *T. cobbi*, with lesser but still significant values between insular and continental populations of *C. platensis* and *T. falcklandii* (Table 1). From these surveys, we chose the pair with the highest divergence, the wrens *T. aedon* and *T. cobbi*, and one of the species showing lower genetic differentiation but larger sample size, *T. falcklandii*, to conduct further analyses.

The divergence in COI observed between *T. aedon/T. cobbi* and within *T. falcklandii* was confirmed using three additional genes: cyt *b* (mitochondrial), FGB and CHD1 (both nuclear). For cyt *b*, *T. cobbi* haplotypes could not be connected by 95% probability linkages to those of individuals from *T. aedon* and were thus assigned to a separate network (see Supporting information, Fig. S1). Insular *T. falcklandii* populations showed private cyt *b* haplotypes, differing by one mutational step from continental ones (see Supporting information, Fig. S1). Overall Φ_{ST} and K2P estimates for the cyt *b* data are consistent with the degree of divergence observed in COI for *T. aedon/T. cobbi* and within *T. falcklandii* (Table 1). High and significant Φ_{ST} values were also obtained using CHD1 sequences and its inferred haplotypes, as well as inferred haplotypes from the 3′ region of FGB (Table 2), confirming that the genetic pattern seen between *T. aedon* and *T. cobbi* is not limited to mitochondrial genes. *T. falcklandii* continental and

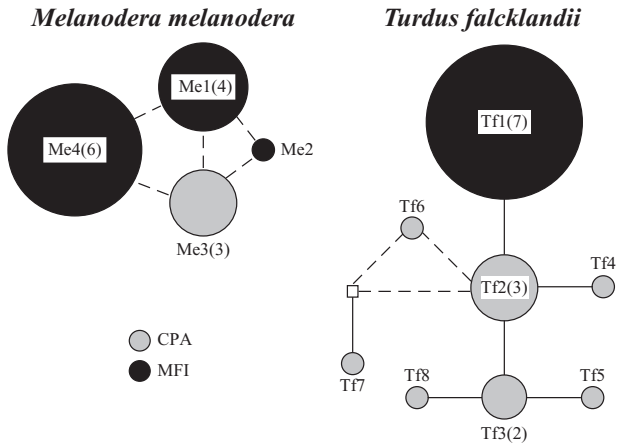

Figure 3. Unrooted maximum parsimony network showing 95% probability linkages among cytochrome *c* oxidase I (COI) haplotypes obtained from *Melanodera melanodera* and *Turdus falcklandii*. Other details are as provided in Fig. 2.

insular populations did not show statistically significant differences in the nuclear loci assayed (Table 3), possibly a consequence of lower mutation rate, large effective population size and incomplete lineage sorting for these biparentally-inherited, diploid markers.

The divergence time between insular and continental populations was estimated using cyt *b* data and a 2.1% divergence per million year calibration (Weir & Schluter, 2008). Time to most recent common ancestor (TMRCA) between *T. aedon* (samples from network A; Fig. 4) and *T. cobbi* was approximately one million years [95% confidence interval: 415 400 to 1 858 700 years before present (BP)]. The inferred split between *T. falcklandii* populations occurred more recently (44 500 years BP, 95% confidence interval: 645–112 000 years). Our IMa2 simulations did not fully resolve all parameters in the model: neither the *T. aedon/T. cobbi*, nor the *T. falcklandii* datasets allowed for precise estimation of ancestral effective population sizes and splitting times; nor did the

Table 1. Pairwise Φ_{ST} values and average Kimura two-parameter (K2P) distances between Malvinas – Falkland Islands (MFI) and continental populations calculated using either cytochrome *c* oxidase I (COI) or cytochrome b (cyt *b*) sequences

	COI					Cyt *b*				
Species	Φ_{ST}	*P* value	% K2P	SD	*N*	Φ_{ST}	*P*	% K2P	SD	*N*
*Troglodytes aedon/cobbi**	0.89	< 0.0001	2.17	0.13	18-11	0.67	0.022	2.86	0.36	5-3
*Cistothorus platensis**	0.58	0.035	0.55	0.58	4-4	–	–			–
Turdus falcklandii	0.52	< 0.0001	0.28	0.12	10-7	0.83	0.014	0.14	0.08	5-5
Melanodera melanodera	0.23	0.232	0.15	0.10	3-11	–	–			–
Carduelis barbata	–0.02†	0.540	0.17	0.18	19-10	–	–			–

*For these calculations, we only used samples from network A.
†Interpret as 0.
Only those species for which more than two samples were available from either population were included in the analysis. Sample sizes (*N*) correspond to continental and island populations respectively. Φ_{ST} comparisons are accompanied by their respective *P*-values and, for K2P distances, SD are informed.

Table 2. Pairwise Φ_{ST} comparisons and Kimura two-parameter (K2P) distances between the wrens *Troglodytes cobbi* and *Troglodytes aedon* (network A) obtained using chromodomain-helicase-DNA binding protein (CHD1) and β-fibrinogen gene (FGB) sequences as well as haplotypes inferred for the 5′ and 3′ portions of each loci

Marker	Φ_{ST}	*P*	% K2P	SD	*N*
CHD1	1	< 0.0001	0.42	0.10	7–9
CHD1 5′ haplotypes	0.93	< 0.0001	0.55	0.17	12–16
CHD1 3′ haplotypes	0.65	< 0.0001	0.85	0.36	10–18
FGB	0.30	0.079	0.22	0.25	7-5
FGB 5′ haplotypes	0.26	0.089	0.67	0.35	6-6
FGB 3′ haplotypes	0.51	0.010	0.59	0.36	4–8

Other details as in Table 1.

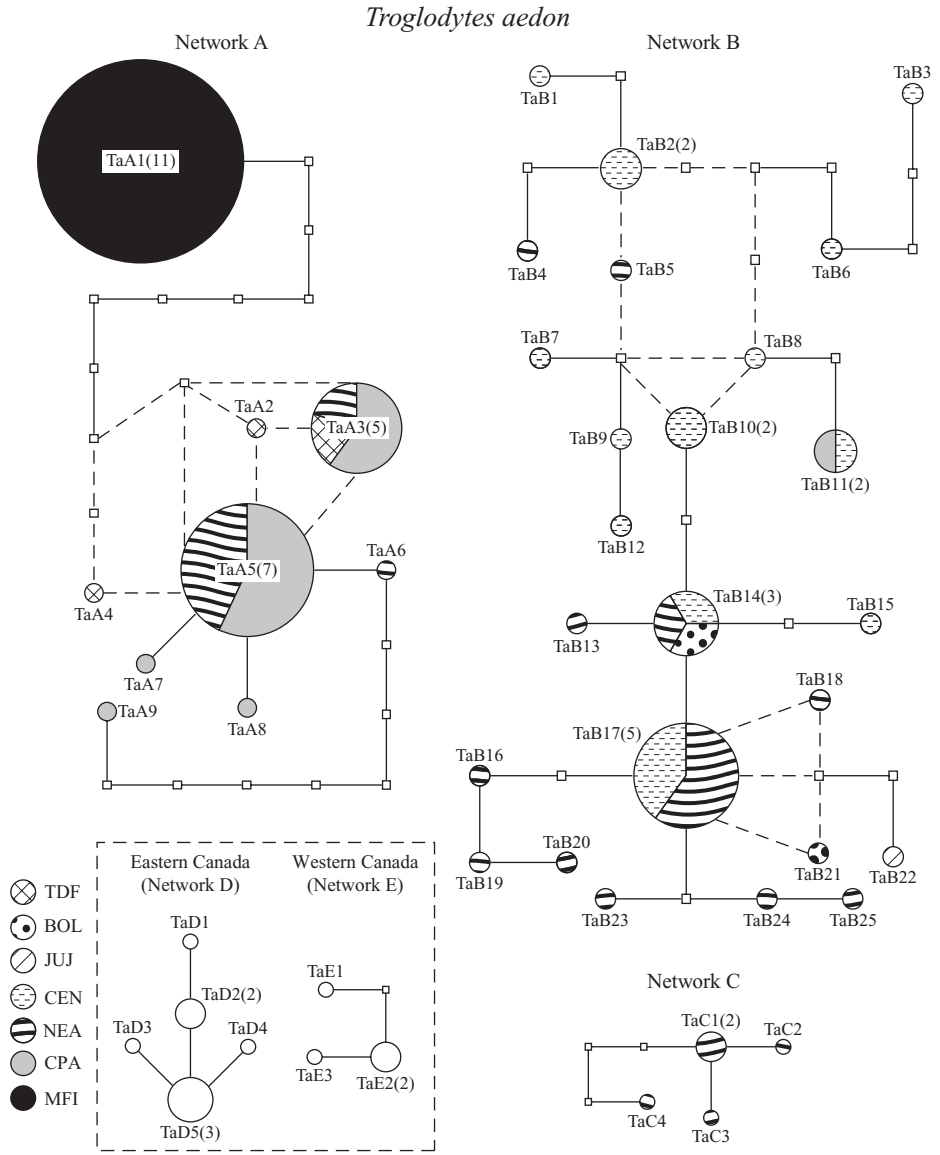

Figure 4. Unrooted maximum parsimony networks showing 95% probability linkages among cytochrome *c* oxidase I (COI) haplotypes obtained from *Troglodytes aedon* and *Troglodytes cobbi*. Note that five different networks (A to E) were obtained in the TCS analysis, with *T. cobbi* haplotypes found within network A. Other details are as provided in Fig. 2.

T. falcklandii dataset resolve the continental effective population size. However, it was possible to obtain estimations of migration in both directions during the divergence between MFI and continental populations. The effective number of migrants per generation (population migration rates, $2N_1m_1$ and $2N_2m_2$) between *T. aedon* and *T. cobbi* and *vice versa* was low or negligible, with estimates of less than one individual per generation and the 95% high posterior density (HPD) interval including zero. For *T. falcklandii*, the mean values were between one and two individuals per generation, although, again, the 95% HPD intervals overlapped with zero.

Phylogeographical structure within continental populations of *T. aedon* and *C. platensis*

Our COI survey also identified divergent and potentially reproductively isolated lineages within continental populations of *C. platensis* and *T. aedon*. *Cistothorus platensis* COI haplotypes were assigned by the TCS analysis to two different networks (Fig. 2): one including individuals from Patagonia and the MFI and the other individuals mainly from Jujuy, in Northwestern Argentina (but also one Patagonian individual). These two lineages differed by

Table 3. Pairwise Φ_{ST} comparisons and Kimura two-parameter (K2P) distances between *Turdus falcklandii* continental and island populations obtained using chromodomain-helicase-DNA binding protein (CHD1) and β-fibrinogen gene (FGB) sequences as well as inferred haplotypes

Marker	Φ_{ST}	*P* value	% K2P	SD	*N*
CHD1	0.25	0.266	0.06	0.10	9-3
CHD1 haplotypes	0.2041	0.070	0.09	0.12	16-6
FGB	0	0.999	0.00	0.00	7-6
FGB haplotypes	0.0875	0.202	0.30	0.33	14-12

Other details as in Table 1.

Table 4. Pairwise Φ_{ST} comparisons and average Kimura two-parameter (K2P) distances between *Troglodytes aedon*/*Troglodytes cobbi* samples belonging to different cytochrome *c* oxidase I networks (Fig. 4)

	Cobb's	A	B	C	D	E
Cobb's		< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.003
A	0.89 (2.17 ± 0.13)		< 0.0001	< 0.0001	< 0.0001	< 0.0001
B	0.82 (4.02 ± 0.36)	0.83 (3.63 ± 0.45)		< 0.0001	< 0.0001	< 0.0001
C	0.97 (5.06 ± 0.22)	0.93 (4.59 ± 0.24)	0.82 (4.13 ± 0.26)		< 0.0001	0.013
D	0.98 (4.28 ± 0.18)	0.94 (4.29 ± 0.23)	0.84 (4.01 ± 0.36)	0.94 (4.53 ± 0.24)		0.001
E	0.99 (6.04 ± 0.39)	0.95 (5.93 ± 0.46)	0.87 (5.44 ± 0.34)	0.95 (5.92 ± 0.23)	0.96 (5.75 ± 0.208)	

Network A was divided into Cobb's wren and the remaining members (noted as A) for this analysis. Φ_{ST} calculations are shown (lower left) with their respective *P*-values (upper right). K2P values (% differences) and their SDs are shown in parenthesis (lower left).

6.71 ± 1.49% K2P distance ($\Phi_{ST} = 0.92$; $P < 0.001$) and it is possible that they are sympatric because different individuals from the province of Río Negro (Argentina) were assigned to different networks. The TCS analysis assigned continental populations of *T. aedon* to three different COI haplotype networks (Fig. 4). Haplotypes from the three networks differ markedly (Table 4), showing mean K2P distances comparable to divergence between southern South America and samples from Canada, on the opposite extreme of this species' distribution. The three lineages are sympatric in Northeastern Argentina, with localities such as Mburucuyá National Park (in the province of Corrientes, Argentina) containing representatives of all three lineages. All Patagonian individuals (CPA and TDF) were assigned to network A. Note that, although one individual belonging to CPA was assigned to network B (Fig. 4), this sample was from an unknown locality in Argentina and thus for analysis we assigned it to the geographical centre of the country (which is in CPA). We included this individual because it was relevant to our comparison between *T. aedon* and *T. cobbi*, although it was excluded when analyzing the continental lineages of *T. aedon*. Finally, the five haplotype networks (including those from eastern and western Canada) formed highly supported and monophyletic clades in a COI Bayesian 50% majority rule consensus tree (see Supporting information, Fig. S2), although basal relationships were not well resolved. Clade A was composed of reciprocally monophyletic sister clades: one including the representatives from *T. cobbi* and one with all continental members.

Song differences between *T. aedon* and *T. cobbi*

Both wrens, *T. aedon* and *T. cobbi*, have highly complex songs, alternating unique syllable types with long sequences of repeated syllables (Fig. 5). We observed striking diversity in vocalizations within *T. aedon*; however, because we obtained samples for genetic analyses and song recordings separately, we could not determine whether song differences were consistent with the different genetic lineages identified. Therefore, with the objective of testing for differences in vocalizations between *T. cobbi* and *T. aedon*, we compared the former from MFI with either all recordings available from *T. aedon* or exclusively to putative members of its sister clade comprised of all the Patagonian *T. aedon* individuals (see Supporting information, Fig. S2). We found significant differences between variables measured from *T. cobbi* songs ($N = 16$) and either continental *T. aedon* ($N = 21$) or only Patagonian populations of this species ($N = 10$). The former comparison differed

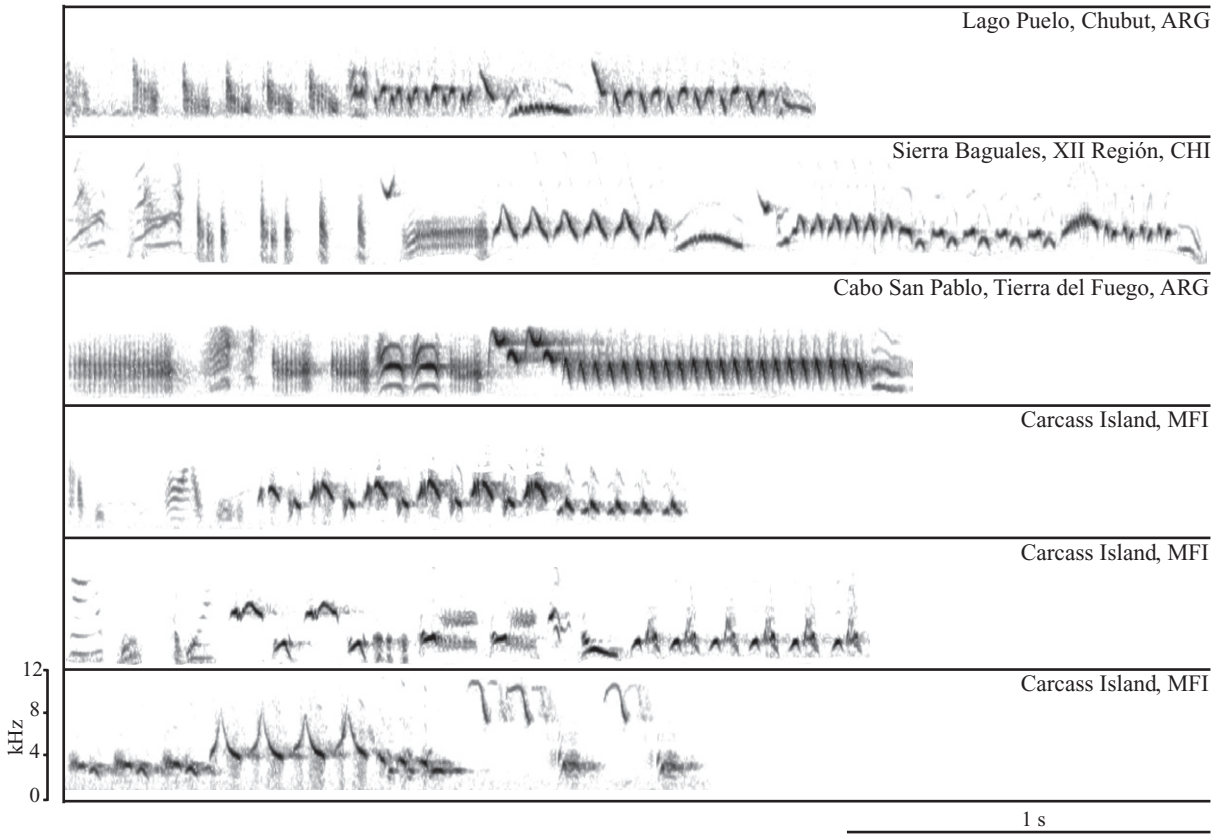

Figure 5. Examples of sonograms from songs of three *Troglodytes aedon* and three *Troglodytes cobbi* males.

significantly in *Min* ($t = -2.55$, d.f. = 27.14, $P = 0.017$); *SD freq* ($t = 2.07$, d.f. = 23.42, $P = 0.050$); and *Trans sec*$^{-1}$ ($t = -2.15$, d.f. = 34.73, $P = 0.039$). When only Patagonian populations of *T. aedon* were considered, *T. cobbi* songs differed significantly in *Percent blank* ($t = -2.23$, d.f. = 15.29, $P = 0.041$); and *Trans sec*$^{-1}$ ($t = 4.94$, d.f. = 22.91, $P < 0.0001$). A DFA (entering variables simultaneously) identified significant differences between *T. cobbi* and continental *T. aedon* (Wilk's $\lambda = 0.591$, $\chi^2 = 16.542$, d.f. = 7, $P = 0.021$), retaining all seven variables and assigning 67.6% of songs to their correct group (five continental and seven insular songs classified incorrectly). When *T. cobbi* was compared with Patagonian *T. aedon* alone, the DFA retained all seven variables and found significant differences between the two groups (Wilk's $\lambda = 0.33$, $\chi^2 = 22.745$, d.f. = 7, $P = 0.002$). Reclassification success was 76.9%, with two continental and four insular songs assigned to the wrong group.

DISCUSSION

The role of the MFI in isolating passerine populations

We surveyed all nine of the regularly breeding passerine species of the MFI, observing mitochondrial DNA patterns that coincide with the morphological subspecies (or putative species) only in four cases. Genetic differentiation between insular and continental populations ranged from negligible (*C. antarcticus*, *M. maclovianus*, *A. correndera*, *S. loyca*, and *C. barbata* all had shared COI haplotypes), to slight (*C. platensis*, *T. falcklandii* and *M. melanodera*), and finally to marked genetic divergence between *T. aedon* and *T. cobbi*. If we assume that the nine passerine species do not differ in their generation time and that the COI mutation rate is relatively constant across species (and similar between COI and COII so that we may include *C. antarcticus*), various processes likely underlie these genetic patterns. Each species could be experiencing different levels of migration, differ in their effective population sizes or have successfully colonized the islands at different times.

Perhaps as a result of the strong prevailing westerly winds (the 'furious fifties' as circumpolar winds are known in the latitudes 50–60 degrees South), various common passerines from continental Patagonia are seen in the MFI as irregular vagrants but these do not reproduce in the islands (e.g. *Mimus patagonicus*, Lafresnaye & d'Orbigny 1837; *Phrygilus fruticeti*, (Kittlitz 1833); *Sicalis lebruni*, (Oustalet 1891); *Zonotrichia capensis*, Muller 1776) (Woods &

Woods, 2006). This suggests that movement or downwind drift from the continent to the islands is not a rare event, and that not all individuals that arrive are able to find breeding partners, locate suitable habitat or adapt to reproduce in the local climatic conditions. We found species in which individuals from the MFI and the continent did not share mtDNA haplotypes (*M. melanodera*, *T. aedon/T. cobbi* and *T. falcklandii*), suggesting that these populations are not experiencing immigration. Because we cannot detect migrants that do not interbreed with the local populations, it remains to be determined whether these species experience lower levels of immigration into the islands than the remaining taxa (perhaps as a result of more sedentary habits; for *T. aedon*, see Kroodsma & Brewer, 2005), or if immigrants are arriving and not reproducing because they are maladapted or as a result of the existence of reproductive isolating barriers. Our IMa2 estimates of the effective number of migrants per generation since the split between populations cannot be distinguished from zero for *T. falcklandii* and for *T.aedon/T. cobbi*. This suggests that migration during the divergence between MFI and continental populations of these species has been extremely low. Moreover, both *T. falcklandii* and *T. aedon* continental populations showed various divergent COI haplotypes but only a single insular one. This is consistent with these taxa having undergone a bottleneck that reduced genetic diversity in the MFI, comprising a founder effect that would result from the colonization of the islands by a small group of continental individuals. Although our sample sizes from the MFI and continent were not equal, with our MFI samples being collected almost entirely from a single island, passerine populations of other species from the former showed comparatively fewer haplotypes (between one and three per species differing by a maximum of only two mutational steps). More sampling in the MFI will confirm whether this apparent founder effect applies to all passerine populations in the archipelago.

Species could also differ in the time at which they effectively established breeding populations in the islands, and indeed this appears to be the case for *T. falcklandii* and for the pair *T.aedon/T. cobbi*. Our estimations suggest that the split between *T. cobbi* and *T. aedon* is an order of magnitude (approximately 20-fold) older than that between continental and insular populations of *T. falcklandii*. It is important to note that, among the species that shared haplotypes between the MFI and the continent, insular representatives of *A. correndera*, *C. barbata*, *M. maclovianus*, and *S. loyca* possess the most abundant continental haplotype. For *C. antarcticus*, this cannot be evaluated because only one continental sample was included. This pattern of shared haplotypes is consistent with a combination of high levels of ongoing gene flow and extremely recent isolation (i.e. retention of ancestral polymorphism) between populations of these species.

Overall, our data suggest that the MFI were not colonized at the same time by the nine passerine species that comprise the land avifauna of the islands. We have shown that *T. cobbi* is the oldest resident MFI land bird, splitting from continental *T. aedon* during the early Pleistocene. *Turdus falcklandii* is much younger, having colonized the islands during the period since the late Pleistocene. This is possibly also the case for the remaining seven species; however, because migration was not quantified, we cannot discard the idea that high levels of gene flow have erased information on the colonization history of the MFI. Thus, founding events at different periods in time could account for the observed variation in genetic patterns across species. Interestingly, the colonization of the MFI that led to the *T. cobbi* lineage dates to approximately one million years, coinciding with a period in which Patagonia was covered by extensive ice sheets known as the Great Patagonian Glaciation (Rabassa, Coronato & Martínez, 2011). It is possible that ocean ice increased connectivity with the continent and that the oceanic climate of the MFI provided refuge for the colonizing individuals. It is hard to explain why, under putative conditions of higher connectivity, the MFI served as a refuge for wren populations only, although it is possible that other species colonized the islands and subsequently became extinct. Detailed sampling of insular populations across a variety of species (not only birds), together with a multilocus phylogeographical approach that allows inferences about demographic history, may help provide a clearer understanding of how and when the MFI were colonized.

Implications for speciation and taxonomy of *C. platensis* and *T. aedon*

Introduced rodents have had a devastating effect on *T. cobbi* (Hall *et al*., 2002; Hilton & Cuthbert, 2010. This taxon is considered Vulnerable to extinction (BirdLife International, 2011), with an estimated 4000–8000 remaining breeding pairs and a range restricted to peripheral islands, where exotic mammals (*Felis silvestris catus*, Schreber 1775; *Rattus norvegicus*, Berkenhout 1769; *Rattus rattus*, Linnaeus 1758; and *Mus* sp., Linnaeus 1758) have not been introduced and the habitat is less degraded by grazing livestock (Woods, 1993; Kroodsma & Brewer, 2005). Hence, resolving the taxonomic status of *T. cobbi* is of particular interest to guide future conservation efforts. We found *T. cobbi* to be monophyletic at mitochondrial DNA loci (COI and cyt *b*) with

respect to the closest sister *T. aedon* clade (see Supporting information, Fig. S2). Φ_{ST} values calculated using both mitochondrial and nuclear DNA (autosomic and Z-linked) are consistent with absence of gene flow. Furthermore, we find significant differences in vocalizations between continental and island taxa. Previous studies have found these two putative taxa to differ also in morphology, plumage, behaviour and ecology (Woods, 1993; Kroodsma & Brewer, 2005). Altogether, the evidence suggests that *T. cobbi* constitutes an independent evolutionary lineage from *T. aedon*.

A mitochondrial survey carried out by Kerr *et al*. (2009) in southern South America compared COI sequences obtained from 500 species of Argentine birds, finding only six species with a maximum 'intraspecific' divergence over 4% K2P distance (*Cinclodes fuscus*, Vieillot 1818; *C. platensis*, *Myiophobus fasciatus*, Muller 1776; *Thamnophilus ruficapillus*, Vieillot 1816; *T. aedon*; and *Upucerthia dumetaria*, Geoffroy Saint-Hilaire 1832). *Upucerthia dumetaria* and *C. fuscus* were found to comprise more than one lineage deserving full species status after more detailed study (Areta & Pearman, 2009; Sanín *et al*., 2009). Two allopatric lineages were found within continental *C. platensis* and three allopatric/parapatric lineages in continental *T. aedon* (Kerr *et al*., 2009). Our COI survey increased the sampling intensity for these two species and identified three highly divergent, and at least partially sympatric, continental lineages within mostly Argentine individuals of *T. aedon* (a few samples from Bolivia were also included), and two highly divergent lineages within continental populations of *C. platensis* that appear to be sympatric. The average COI K2P distance between the two *C. platensis* lineages is 6.71%, higher than any average or even maximum intraspecific comparison obtained before for Argentine species (Kerr *et al*., 2009; Campagna *et al*., 2010). Average values for *T. aedon* were approximately 4%, on the same order of those reported by Kerr *et al*. (2009).

Cistothorus platensis is widespread across North and South America and is currently composed of 20 recognized subspecies (Kroodsma & Brewer, 2005). In the present study, we find evidence of modest isolation between the MFI subspecies *C. p. falklandicus* and continental individuals, although we also observe striking divergence within this taxon that does not correspond clearly with any of the limits between the described subspecies.

The house wren species complex includes three continental forms (one in North America, one in the southern USA and Mexico, and one in South America) and three insular forms (two in islands off the coast of Mexico, and *T. cobbi* in the MFI) that have been split into separate species or alternatively considered conspecific by different studies (Kroodsma & Brewer, 2005). In turn, each continental form includes several subspecies, adding up to 30 in total. We find *T. cobbi* to be highly divergent in COI with respect to continental individuals, as well as three distinct COI lineages within Argentina, showing divergence that is similar in magnitude to comparisons between North American and South American clades. The *T. cobbi* lineage can be diagnosed using mitochondrial and nuclear markers, suggesting it could be considered a separate species from *T. aedon* under the Phylogenetic Species Concept (Cracraft, 1983). Moreover, the vocalization differences observed could reflect differences in mate recognition systems. It remains to be determined (e.g. through playback experiments) whether these differences are sufficient to cause reproductive isolation, thus also justifying species status for *T. cobbi* under the Biological Species Concept (Mayr, 1982). Given the relationship between *T. cobbi* and the different *T. aedon* clades, it follows that *T. aedon*, as currently defined, is a paraphyletic taxon.

Our findings highlight some profitable areas for future research in the MFI; in particular, studies that aim to understand the divergence (or lack thereof) in mate recognition systems and the evolution of ecologically adaptive differences between island birds and the continental populations from which they derive. Moreover, broad comparative phylogeographical studies will help recognize whether the MFI were a Pleistocene glacial refugium and provide insights on the role of the glaciations in facilitating island colonization. Finally, continental-scale phylogeographical studies of both *C. platensis* and *T. aedon* are required to understand the complex genetic patterns we have identified, and to investigate the existence of cryptic species within these taxa.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Unrooted maximum parsimony network showing 95% probability linkages among cyt *b* haplotypes obtained from *Troglodytes cobbi/Troglodytes aedon* and *Turdus falcklandii*. For *T. aedon*, we only included individuals from network A (Fig. 4) because these were the closest relatives to *T. cobbi*. Other details are as provided in Figure 2.
Figure S2. Cytochrome *c* oxidase I Bayesian 50% majority rule consensus tree with posterior probabilities indicating node support. Support for some nodes was omitted for simplicity. For *Troglodytes aedon*, networks A to E from Figure 4 are indicated.
Table S1. Individuals used for genetic analyses. GenBank Accession numbers are provided, as well as cytochrome *c* oxidase I (COI), COII and cytochrome *b* haplotypes.
Table S2. Primers and PCR conditions used to amplify loci in the present study.
Table S3. Recordings used in this study to assess song differences between *Troglodytes aedon* and *Troglodytes cobbi*.

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