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Laying the ground for the evolutionary adaptive conservation of the endangered Darwin's fox (*Lycalopex fulvipes*)

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ABSTRACT

Darwin's fox (*Lycalopex fulvipes*) is one of the most narrowly distributed and endangered carnivores in the world. Until new records began to be collected in 2012, the species was known to occur only on Chiloé Island and the Nahuelbuta mountain range on the mainland. Here, we genotype the partial mitochondrial DNA control region, 11 microsatellite loci, and exon 2 and exon 3 of major histocompatibility complex class II to assess the genetic structure and variation of Darwin's foxes ($n = 105$) from all known localities, with 99 samples collected from the previously known populations and six from new localities where the species has been recorded. We found low genetic diversity, with six mitochondrial DNA haplotypes that were unevenly distributed across the population, strong microsatellite fixation and only one and two polymorphic loci in nuclear genes, respectively, raising major concern regarding susceptibility to infectious diseases. Genetic diversity was markedly lower on Chiloé than on the mainland, which hints at an interplay of founder effect and lack of gene flow following isolation other than small population size. The latter is a concern for the mainland counterpart, where it is compounded by high fragmentation. Since genetic data qualify these two populations as separate management units, conservations actions should be envisaged pertinently. Restoring and further enhancing the connectivity of mainland population is a priority. Based on the findings of this study, a reassessment of its IUCN conservation status, which is currently "Endangered", might be urgently needed.

1. Introduction

Achieving a comprehensive understanding of spatiotemporal genetic variation in threatened species is key to implementing effective conservation management strategies (Kardos et al., 2021). Assessing the degree and extent of genetic diversity is fundamental for preserving

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inter- and intra-population variability, as well as population distinctiveness, by defining management units within an adaptive evolutionary conservation framework (Fraser and Bernatchez, 2001). The growing recognition that sustained wildlife monitoring efforts are needed not only to maintain but also to restore genetic diversity is reflected in an increasing number of international agreements and mandates that explicitly include this objective in their agendas (Forcina and Leonard, 2020). Over the past few decades, a range of technological and organizational advancements has significantly facilitated the integration of genetic diversity—long neglected in this context—into the goals of global conservation policies (Hoban et al., 2021). Efforts are now underway to translate this progress into coordinated action plans at national, subnational, and supranational levels (Hoban et al., 2024). Nevertheless, several narrowly distributed and highly endangered species still lack comprehensive assessments of population genetic structure, underscoring an urgent need for action. As a case in point, the conservation management of Darwin's fox (*Lycalopex fulvipes* Martin, 1837) (Fig. S1; Videos S1 and S2), one of the rarest and least known canids (Macdonald and Sillero-Zubiri, 2004), is constrained by the absence of a comprehensive assessment of its population structure. Currently classified as "Endangered" by the International Union for the Conservation of Nature (IUCN) (2004), this species has long been known to occur solely in the forested areas of Chiloé Island, along the coast of southern Chile. However, a mainland population was discovered in the Nahuelbuta mountain range at the end of the previous century (Medel et al., 1990), a finding later confirmed by mitochondrial DNA analyses (Yahnke et al., 1996). Subsequently, a Darwin's fox hide was recovered in Punta Chanchán (Los Ríos Region) (Vilà et al., 2004) and found to hold the same control region haplotype as some individuals from Nahuelbuta, which was therefore guessed to be its population of origin. However, from 2012 onwards, new mainland records have been reported through roadkills—such as the one genetically identified in Gorbea (D'Elia et al., 2013)— and via camera trap data from locations including Oncol and the Valdivian Coastal Range (Farias et al., 2014; Silva-Rodríguez et al., 2016). These findings have led to an updated range for Darwin's fox, extending from Chiloé Island in the Los Lagos region (43.4°S) northward to the southern border of the Biobío region (37° 48′S) (Fig. S2). Nevertheless, this small-sized fox still has one of the narrowest distributions among all extant canids and is also one of the few terrestrial mammals endemic to the Valdivian Rainforest Ecoregion—one of the World biodiversity hotspots (Myers et al., 2000)—along with the long-nosed caenolestid (*Rhyncholestes raphanurus*) (Martin, 2011), Mann's soft-haired mouse (*Abrothrix manni*) (D'Elía et al., 2015), and microbiotherids (*Dromiciops* spp.) (D'Elía et al., 2016). The largest known Darwin's fox population, inhabiting Chiloé Island, was estimated at a minimum of 412 mature individuals, whereas this figure drops to about 227 individuals in Nahuelbuta (Silva-Rodríguez et al., 2016). When the latest new records and potential areas of species distribution were considered, the minimum size of the continental population was estimated to be about 489 individuals (Silva-Rodríguez et al., 2016). These low numbers—compounded by the physical isolation of the two populations—expose Darwin's fox to a high extinction risk due to infectious diseases (Di Cataldo et al., 2020; Hidalgo-Hermoso et al., 2022) and habitat loss (Silva-Rodríguez et al., 2016). Other threats include attacks by stray dogs—which also can transmit diseases (Hidalgo-Hermoso et al., 2020)—or intraguild predation by pumas (*Puma concolor*) and other larger sympatric fox species, such as the South American grey fox or chilla fox (*L. griseus*) and the culpeo or Andean fox (*L. culpaeus*) fox (Jiménez, 2000), which are also reported to competitively exclude their smaller congener (Vilà et al., 2004).

On Chiloé Island, Darwin's fox occurs mainly in the western sector, where pristine forests persist despite increasing firewood exploitation, but has also recently been recorded in the northern part of the island (near Ancud; E.H.H., personal communication) The local population of this canid has been proposed to be larger yet more genetically depleted than its mainland counterpart (Vilà et al., 2004); however, this assumption remains unproven. To date, there is no comprehensive genetic study covering all Darwin's fox populations across the species' known range.

To fill this knowledge gap and inform the conservation strategies of this rare species at the national and international levels, we here use mitochondrial and nuclear DNA analyses to 1) assess neutral and adaptive genetic diversity across all extant Darwin's fox populations, 2) infer their phylogenetic relationships and population genetic structure, and 3) identify management units for conservation.

2. Material and methods

2.1. Sample collection and DNA extraction

Between 2009 and 2017, we conducted 35 expeditions lasting four to fifteen days across 22 and 9 localities on Chiloé Island and continental Chile, respectively, and collected 121 Darwin's fox samples from Chiloé, Nahuelbuta, Oncol, the Valdivian Coastal Reserve (VCR), and Gorbea (Fig. S2). In addition, 19 DNA samples from Nahuelbuta, collected by E. MacMahon and W.E.J. between 1998 and 2002, were included in our dataset (Table S1). For blood sample collection, foxes were captured using box traps set in the evening and checked at dawn. The animals were anaesthetized with a either a combination of 10 mg/kg of ketamine (Imalgene, Merial, France) and 1 mg/kg of xylazine (Xilacina, Centrovet Ltda., Chile) or 0.04 mg/kg of dexmedetomidine (Dexdomtor, Pfizer, Germany), which was reversed with 0.4 mg/kg of atipamezol (Antisedan, Pfizer, Germany). Under anesthesia, each fox underwent a basic external clinical evaluation by a veterinarian before 4 ml of blood was collected from the cephalic vein. All live animals were microchipped (EI1001, Felixcan Animal ID) subcutaneously and released in the same site of capture. Hair samples ($n = 2$) were collected from the tail and from box traps visited by foxes without unsuccessful capture. Fresh scat samples ($n = 35$) were collected along trails. Skin samples were obtained from taxidermized animals preserved in Nahuelbuta National Park ($n = 2$), in the Chilean National Museum of Natural History in Santiago ($n = 1$), and from a pelt that was kept in a farmer's house ($n = 1$). Finally, one muscle sample was collected from a fox killed by a dog in Comuna de Gorbea. Each sample was placed in 2 ml vials containing absolute ethanol and stored at −20 °C until DNA extraction. All sampling was conducted under capture and sampling permits from the National Agricultural and Livestock Service (SAG), authorization numbers 1262/2009, 2263/2010, 206/2012, 3155/2013, 3363/2015, 3035/2016, and 5029/2017

Total genomic DNA was isolated from blood and muscle samples by proteinase K digestion, followed by DNA extraction with phenol/chloroform protocol (Sambrook et al., 1989). DNA from feces was extracted using the QIAamp DNA Stool Kit (Qiagen, Hilden, Germany), while DNA from skin and hair samples using the QIAamp DNA Investigator Kit (Qiagen), following the manufacturer's instructions in a dedicated room.

2.2. Gene amplification and sequencing

We amplified the partial mitochondrial control region (CR) gene (partial sequence: 599 bp; nt. positions 15,420–16,018 of *Lycalopex* sp. genome reference MN181407.1: Popović et al., 2020). Amplification was performed using primers MTLPRO2 and CCR-DR1 (Tchaicka et al., 2006) or, for degraded DNA samples, with newly designed internal primers: LfuCR-R1 5′-TCTCAAATGGGACATCTCGA-3′, LfuCR-F1 5′-CCCCCGAAAATTAAAAGATACC-3′, LfuCR-R2 5′-AGGGATTAATCAC-CATGCCTC-3′, and LfuCR-F2 5′-CGGAGCGAGAAGAGRGGCAT-3′. Extraction and PCR blanks were included in each experiment to check against possible contamination. PCR products were treated with 0.5 U of alkaline phosphatase and 4 U of exonuclease prior to bidirectional sequencing using the BigDye Terminator Cycle Sequencing Kit 3.1 on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems/Hitachi, Waltham, USA).

2.3. Sequence data analysis

The newly generated sequences were manually edited in BioEdit 7.0.5 (Hall, 1999) and aligned in ClustalX 2 (Larkin et al., 2007). Previously obtained Darwin's fox sequences (Yahnke et al., 1996; D'Elia et al., 2013) were incorporated in the CR alignment but excluded from the phylogenetic analysis because they were 344-bp and 401-bp long, respectively, as opposed to the 599-bp long ones obtained in the present study. We collapsed all the sequences into unique haplotypes in Collapse 1.2 (Posada, 2004) and built a median-joining haplotype network (Bandelt et al., 1999) in PopArt (Leigh et al., 2015). Mutated positions were uniformly weighted, and the ε-tolerance parameter and transition/transversion ratio were set to 0 and 1, respectively (Bandelt et al., 1999). Subsequently, we used DnaSP 6 (Rozas et al., 2017) to analyze polymorphic sites and compute the number of haplotypes (K), the number of polymorphic sites (S), nucleotide diversity (π), haplotype diversity (Θ), and the average number of nucleotide differences (Π). Moreover, we used Arlequin 3.5.2.2 (Excoffier and Lischer, 2010) to compute the Mismatch Distribution (MD) of pairwise distances to investigate the historical demography of the mainland individuals only ($n = 30$), due to the uniqueness of the CR haplotype in their island population.

To elucidate the timing of the species diversification process across its range, we first performed statistical coalescent analyses in Beast 2.7.7 (Bouckaert et al., 2019). using GeneBank references JX890316, JX890364, JX890331 and JX890370 (*L. culpaeus*, *L. griseus*, hoary fox *L. vetulus* and Pampas fox *L. gymnocercus*, respectively: Tchaicka et al., 2016) along with KT448284 (Sechuran fox *L. sechurae*: Koepfli et al., 2015) as outgroups. The substitution model was co-estimated with the phylogeny using the bModelTest package implemented in BEAST. A strict molecular clock was applied, and a constant coalescent prior with a lognormal distribution (10% standard deviation) was used to better represent branching times within species. We followed Chavez et al. (2022) in dating the *L. griseus*, *L. culpaeus*, *L. sechurae*, *L. gymnocercus* and *L. fulvipes* clade to 1 million years ago (mya), as inferred from their analyses. We then inferred changes in effective population size for the entire dataset ($n = 99$) using a Bayesian skyline plot (BSP) (Drummond et al., 2005) in Beast 10.4 (Suchard et al., 2018). Analyses were performed under the HKY model, as inferred from Jmodeltest2 (Darriba et al., 2012), with lognormal priors and a mutation rate of 4.27×10^{-8} substitutions/site/year (10% standard deviation), as determined from the coalescent tree analyses. This estimated mutation rate is similar to other established mitochondrial control region (CR) clocks in mammals, such as 5.5×10^{-8} substitutions/site/year) in mammals (Ho et al., 2005). Jmodeltest2 and BSP analyses were run in the CIPRES portal (Miller et al., 2010) by two independent Markov Chain Monte Carlo (MCMC) chains: 15 million generations for phylogeny estimation and 150 million generations for the complete dataset (BSP), sampling every 10,000 generations. Convergence and mixing of independent runs were evaluated by visually inspecting and comparing traces of each statistic and parameter in Tracer 1.7 (http://beast.bio.ed.ac.uk/ tracer). Effective sample size (ESS) values >200 were considered good indicators of parameter mixing. The first 10% of trees were discarded as burn-in. Samples were merged using LogCombiner, and the resulting trees were summarized using TreeAnnotator to generate a maximum clade credibility (MCC) tree with mean node heights (Heled and Bouckaert, 2013). BSP log and tree files were imported into Tracer to generate the plot. Trees were visualized in FigTree 1.4.4 (Rambaut, 2010) and edited in Inkscape (2020) (https://inkscape.org).

2.4. Microsatellite analysis

All samples were genotyped at eleven microsatellite loci isolated from Darwin's fox, following Cabello and Dávila (2014). PCR Fragments were resolved by electrophoresis using a 500 LIZ Size Standard on an ABI PRISM 3130xl Sequencer (Applied Biosystems). To ensure the robustness of the dataset, amplification was repeated twice per locus (Navidi et al., 1992; Taberlet et al., 1996; Bellemain and Taberlet, 2004), and Micro-Checker 2.2.3 (Van Oosterhout et al., 2004) was used to detect allelic dropout, null alleles, and genotyping errors. We tested for deviation from Hardy–Weinberg equilibrium (HWE) and linkage equilibrium at each locus and historically known population (Nahuelbuta and Chiloé) using Genepop 1.2 (Raymond and Rousset, 1995). Deviations from HWE were assessed with a Markov-chain algorithm (Guo and Thompson, 1992), while linkage equilibrium was tested using an exact test (Raymond and Rousset, 1995), with *p*-values adjusted via the sequentially rejective Bonferroni method (Rice, 1989). Allele frequencies, as well as observed and expected heterozygosity, were estimated using GenAlEx 6 (Peakall and Smouse, 2006). Arlequin was employed to estimate the inbreeding coefficient (F_{IS}) and the fixation index (F_{ST}), which quantifies the degree of population differentiation. Allelic richness was subsequently computed in Fstat 2.9 (Goudet, 2001). To assess the genetic affinity of foxes from newly sampled localities (Gorbea, Oncol, and VCR), we performed a factorial correspondence analysis in Genetix 4.04 (Belkhir et al., 2004) and a Bayesian clustering analysis using Structure 2.3.4 (Hubisz et al., 2009). For this purpose, the number of genetically distinct subpopulations (K) was set to range from 1 and 10, with four iterations per K. Simulations were run with 10^6 MCMC iterations and a burn-in of 10^5 iterations, and the independent allele frequencies model was selected. We chose the most reliable K value based on the algorithm implemented in StructureSelector (Li and Liu, 2018), and individuals were unambiguously assigned to a given population using a threshold of $Q_i = 0.90$ (Vähä and Primmer, 2006).

2.5. MHC amplification and sequencing

We amplified exon 2 of the DQB gene (243 bp) and exon 3 of the DRB gene (200 bp) of the major histocompatibility complex (MHC) class II using primers DM-1 and DM-2 (Hedrick et al., 2000) and DQB-F and DQB-R (Fernandez-Vina and Bignon, 1997), respectively. PCR amplifications were performed in a total volume of 20 μl, containing approximately 50 ng of genomic DNA, PCR buffer with 2 mM $MgCl_2$ (Biotools B&M Labs, S.A., Madrid, Spain), 0.1 mM of each dNTPs (Biotools), 0.5 μM of each primer, 0.5 U of Taq DNA polymerase (Biotools), and sterile deionized water. Thermal cycling conditions were as follows: an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at the following temperatures depending on the primer pair—62 °C (MTLPRO2/CCR-DR1), 56 °C (MTLPRO2/LfuCR-R1), 58 °C (LfuCR-F1/LfuCR-R2), 56 °C (LfuCR2/CCR-DR1), 61 °C (DM-1/DM-2), and 58 °C (DQB-F/DQB)—and primer extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. We generated 10 clones per PCR product and individual using the PCR Cloning Kit (Qiagen), and sequenced them using universal M13 primers. For clarity, it should be noted that, in the absence of a reference genome and without prior knowledge of MHC copy number variation in Darwin's fox, some uncertainty remains in these estimates due to the potential amplification of multiple loci.

3. Results

3.1. Mitochondrial DNA variation and historical demography

We successfully amplified CR sequences for 99 of the 121 biological samples (81.8%), with a particularly low success rate for the 35 stool samples (9%). Overall, we identified six CR haplotypes, each private to one of the surveyed localities (Fig. 1a), and observed low mitochondrial diversity (Table S2) despite large pairwise differences among sequences (Table S3).

The most recent common ancestor (TMRCA) of *L. fulvipes* was estimated at 760,000 years ago (median height; 95% HPD: 470,000–1000,000), while the *L. fulvipes* clade population is estimated to have originated 260,000 years ago (95% HPD: 120,000–430,000) before present (Fig. 1b). Divergence between the most recent common

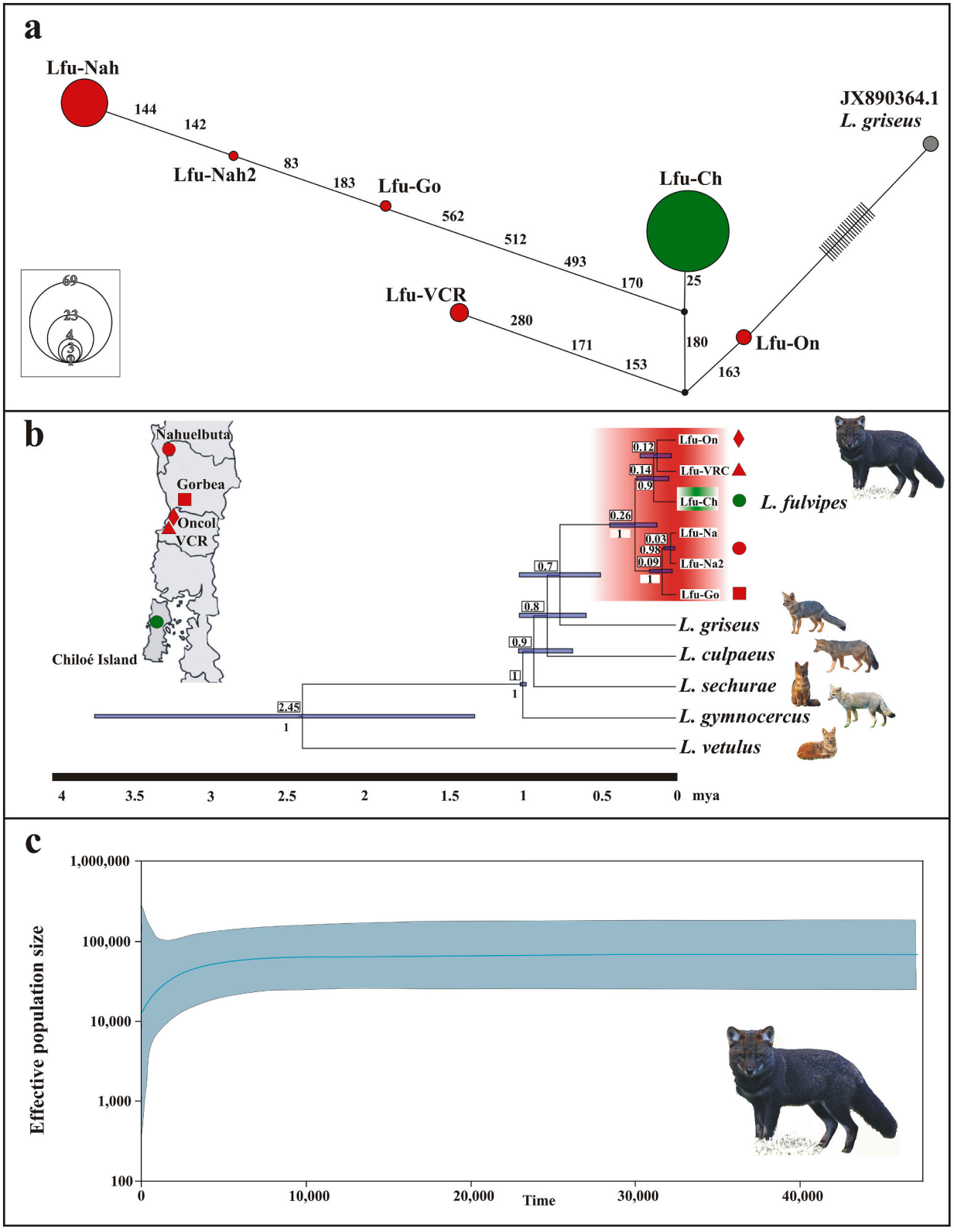

Fig. 1. Mitochondrial DNA results. (a) CR haplotype network based on 99 Darwin's foxes. Each haplotype is indicated by a circle with diameter proportional to the number of sequences. A scale to infer the number of sequences (i.e. individuals) for each haplotype (1–69) is provided; mutational steps are indicated by their nucleotide position or by hatch marks. Individual sequences from island and mainland populations are given in green and red, respectively, while intermediates nodes are given in black. (b) CR Bayesian time tree of *Lycalopex* species as inferred by BEAST. Bayesian posterior probabilities (>0.92) are shown below the nodes, with boxed numbers indicating node ages. Blue bars represent the 95% highest posterior density (HPD) intervals for each node. (c) Bayesian skyline plot of *L. fulvipes* ($n = 99$). Pictures not to scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ancestor of Chiloé and VCR + Oncol foxes is estimated at approximately 140,000 years ago (95% HPD: 40,000–250,000), while Nahuelbuta + Gorbea foxes form a sister clade to this group, with a more recent divergence of 90,000 years ago (95% HPD: 20,000–170,000) (Fig. 1b). The BSP suggests a stable population experiencing a decline during the mid-Holocene (approximately 5000 years before present), and a much steeper decline toward the end of the Holocene (Fig. 1c). The more recent dates inferred from the BSP compared to the phylogenetic tree were expected, as BSP reflects the timing of coalescent events rather than population splits. It begins when lineages start to coalesce, which often occurs long after the initial population divergence. MD analysis on mainland foxes (tau = 11.060), based on the full sequence length (599 bp), a generation time of 3.8-years (Chavez et al., 2022) and a CR mutation rate of 6.08×10^{-7} substitutions/site/year—inferred from the average fossil-calibrated canid Cytochrome-b mutation rate of 2.8% × 10^6 generations (Wayne et al., 1997) and adjusted from the first hypervariable region (HVR I) rate of 1.6×10^{-7} substitutions/site/year (Soares et al., 2009)—suggests an ancient spatial and demographic expansion around 57,000 years ago. However, the high raggedness index (0.3632, $p = 0.86$), e moderate sum of squared deviations (SSD) value (0.02282, $p = 0.53$), and a mismatch distribution with excess differences at 3, 9, and 11 mismatches indicate that the model may not fit the data well.

3.2. Microsatellite variation

We assessed allelic variation at the panel of 11 microsatellites in 64 individuals (Table S4). Three and two loci were found to be monomorphic in Chiloé and Nahuelbuta populations, respectively (Table S5). No evidence of HWE nor linkage disequilibrium emerged. Across all Darwin's fox samples, a total of 50 alleles were identified, with the number of alleles per locus ranging from 1 to 7; private alleles were observed for each sampling locality (Table S6). Allelic richness was higher in Nahuelbuta (3.090) than in Chiloé (2.129). Observed heterozygosity per locus ranged from 0.051 to 0.526 in Chiloé (mean = 0.284) and from 0 to 0.823 in Nahuelbuta (mean = 0.530). F_{IS} was positive in 7 of 11 loci in Chiloé and in 5 of 9 loci in Nahuelbuta. Mean F_{IS} values were also positive, indicating inbreeding effects in both populations The F_{ST} value between Chiloé and Nahuelbuta populations was high (0.448) and statistically significant ($p < 0.05$). Factorial correspondence analysis revealed two main groups: one comprising all foxes from Chiloé and the other including all mainland foxes, within which a subtle substructure based on sampling localities was detectable (Fig. 2a, b). Similarly, Bayesian analysis indicated $K = 2$ as the most supported number of clusters. All Chiloé individuals, except one (Pfu079, $Qi = 0.824$), were unambiguously assigned to cluster I (average $Qi = 0.993$), while all mainland individuals were assigned to cluster II (average Qi ranging from 0.961 to 0.997), with one exception (Pfu072, $Qi = 0.575$) (Fig. 2c).

3.3. MHC variation

Our analysis revealed extremely low MHC variation across all Darwin's fox samples ($n = 99$), with only two DRB alleles detected in Chiloé and Nahuelbuta (Lfu-DRB01 and Lfu-DRB02), which were also present in the other mainland foxes. Notably, Lfu-DRB01 was the predominant allele, occurring in 93.4% of individuals, while four foxes from Nahuelbuta appeared monomorphic at locus Lfu-DRB02 (Table S7). DQB was monomorphic, exhibiting nucleotide differences of 26 and 30 bp relative to Lfu-DRB01 and Lfu-DRB02, respectively. No frameshifts or stop codons were detected, ruling out the possibility that these sequences are pseudogenes. DRB and DQB alleles were compared with homologous sequences from other canids in GenBank to confirm their correspondence to the studied loci. All three MHC alleles were novel among canids. Monomorphism was higher in the Chiloé population than in Nahuelbuta, with 86% and 45% of individuals being monomorphic, respectively. Darwin's fox sequences were submitted to GenBank under accession numbers KM880155 (Lfu-DRB01), KM880156 (Lfu-DRB02), and KM880157 (Lfu-DQB). The primers DQB-F/DQB-R, used to amplify the DQB locus, also amplified Lfu-DRB in 8 of the 99 samples genotyped.

4. Discussion

Evaluating the degree of genetic diversity—and, consequently, adaptive potential—is crucial for the conservation of endangered species (Eizaguirre and Baltazar-Soares, 2014). In this study, we assess the genetic structure and diversity of Darwin's fox, whose population was initially estimated at fewer than 1000 mature individuals (census estimate: Silva-Rodríguez et al., 2018) and later at 5100–5500 individuals (effective population size: Chavez et al., 2022), making it one of the most endangered canids—and arguably one of the most threatened carnivores—on Earth.

4.1. Genetic diversity

The limited mtDNA variation and the strong genetic structure, with private haplotypes, indicate of some degree of isolation and reduced gene flow, in addition to the effects of small population size. Accordingly, the six haplotypes found were associated with low haplotype diversity when compared with the 10 haplotypes found in 66 individuals from 11 populations of another endangered canid, the Ethiopian wolf (*Canis simensis*) (Gottelli et al., 2004). Although Darwin's fox appears abundant in Chiloé, the recovery of only a single haplotype among 69 sampled individuals suggests a possible founder event involving a single mitochondrial lineage. Arguably, this pattern could reflect the limited size of the DNA fragment amplified. However, it aligns with a recent genome-wide study of South American canids, which reported low heterozygosity in Darwin's fox, attributing it primarily to recent inbreeding driven by human-induced habitat loss rather than past climatic events. Similarly, microsatellite diversity was low in both Chiloé and Nahuelbuta populations, with the lowest levels observed on Chiloé Island despite its larger population size. These levels of genetic diversity are comparable to those observed in similarly depleted carnivores that were, until recently, on the brink of extinction. For example, the Iberian lynx (*Lynx pardinus*) exhibits an average observed heterozygosity of 0.387 and an average of 3.75 alleles per locus (based on a panel of 36 loci; Casas-Marce et al., 2013), values comparable to those of the Nahuelbuta fox population and even higher than those recorded on Chiloé. Compared to the Ethiopian wolf, the number of alleles in Darwin's fox is similar to that observed in separate wolf populations analyzed at a panel of nine loci (Gottelli et al., 1994), with average observed heterozygosity being even lower. The strong genetic differentiation between Chiloé and mainland fox populations indicates a lack of gene flow, as expected given their long-term geographic isolation. This pattern aligns with the presence of private alleles, a phenomenon also observed in other carnivores as a consequence of population isolation and inbreeding (e.g. McManus et al., 2015).

At the same time, the limited diversity at the MHC, a key genetic determinant of infectious disease resistance in vertebrates (Bernatchez and Landry, 2003), is particularly concerning. MHC genes typically exhibit high allelic diversity, long-term allele persistence, and elevated heterozygosity (Klein et al., 1993). Our data suggests that Darwin's fox experienced a strong MHC depletion, with only a slight variation of DRB locus between the island and mainland populations DQB locus being monomorphic in the entire sample. Contrasting patterns have been reported in other bottlenecked canid populations: 11 DRB and 9 DQB alleles were found in the Finnish wolf (*C. lupus*), which was driven close to extinction and has limited gene flow from neighboring populations (Niskanen et al., 2014); 9 DRB and 8 DQB alleles in the Italian wolf (*C. l. italicus*) (Galaverni et al., 2013); 17 DRB and 2 DQB alleles in the African wild dog (*Lycaon pictus*) (Marsden et al., 2009); and 4 DRB and 5 DQB alleles in the largest Ethiopian wolf population (Kennedy et al., 2011). On the other hand, polymorphism at the DQB locus has been reported in

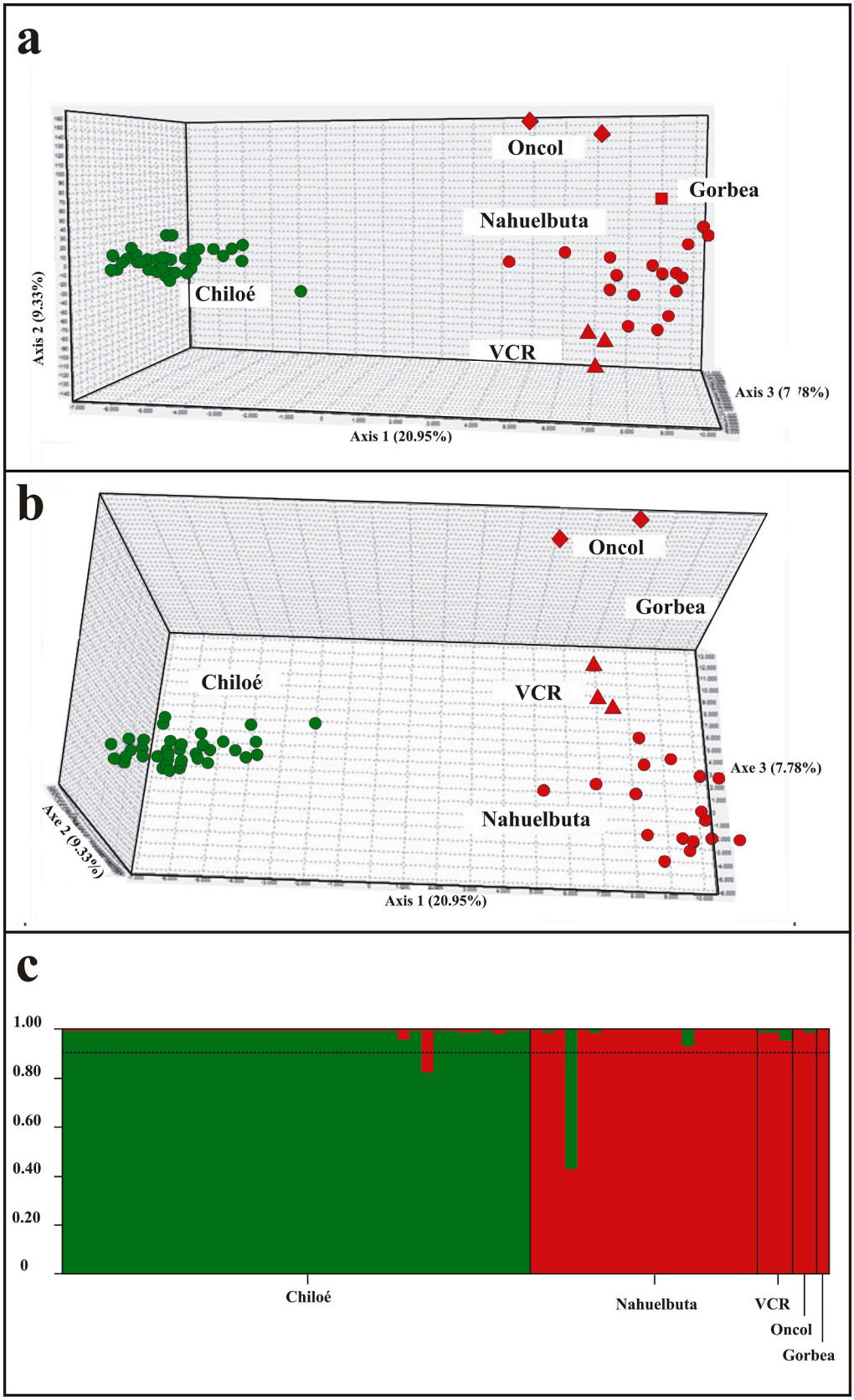

Fig. 2. Microsatellite DNA results. (a, b) Factorial correspondence analysis based on 11 microsatellite loci of the Darwin's fox, with island and mainland populations given in green and red, respectively. (c) Bayesian admixture analysis of Darwin's fox microsatellite genotypes as inferred in Structure ($K = 2$). Individuals are represented as vertical bars partitioned in K segments, the length of which is indicative of the assignment probability to the Kth cluster. Dotted lines mark out the $Qi = 0.90$ threshold value for univocal cluster assignment. VCR: Valdivian Coastal Reserve. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the critically endangered St. Nicholas Island fox (*Urocyon littoralis dickeyi*) (Aguilar et al., 2004), whereas the Mednyi Island population of the Arctic fox (*Vulpes lagopus*) was monomorphic at both DRB and DQB loci (Ploshnitsa et al., 2012).

MHC genes are among the most polymorphic genes in vertebrates (Spurgin and Richardson, 2010), with their exceptionally high variability thought to be maintained by balancing selection imposed by pathogens (Hedrick and Kim, 2000). Nonetheless, in small populations, genetic drift can outweigh the effects of balancing selection (Allendorf and Luikart, 2007). The island and mainland populations of Darwin's fox share the same MHC alleles, potentially reflecting an ancient and severe bottleneck predating their divergence, which may have maintained only a few alleles in the species for several thousand years. Although reduced MHC variation is typically associated with increased susceptibility to infectious diseases (Paterson et al., 1998; Hedrick and Kim, 2000; Lachish et al., 2007), the sampled foxes appeared clinically healthy. Several species that have experienced extreme bottlenecks also show reduced MHC diversity, including the giant panda (*Ailuropoda melanoleuca*) (Zhu et al., 2007), the Canadian mountain goat (*Oreamnos americanus*) (Mainguy et al., 2007), the Scandinavian beaver (*Castor fiber*) (Ellegren et al., 1993), and the northern elephant seal (*Mirounga angustirostris*) (Weber et al., 2004). These populations not only survived but in some cases increased in number, representing rare examples of species recovery following severe genetic depletion (Ellegren et al., 1993; Mainguy et al., 2007). Conversely, reduced MHC diversity can render species particularly susceptible to infectious agents or other diseases. This is exemplified by the Tasmanian devil (*Sarcophilus harrisii*), in which the spread of a transmissible and fatal facial tumor appears to be facilitated by extremely low MHC I variation (Radwan et al., 2010; Siddle et al., 2007). Notably, Darwin's fox exhibits fewer MHC II-DRB alleles and lower nucleotide diversity than cheetahs (*Acinonyx jubatus*), a species long cited as an iconic example of severely depleted MHC diversity. In cheetahs, this reduced diversity is associated with limited immunocompetence and heightened vulnerability to pathogens to which they have not previously been exposed (Castro-Prieto et al., 2011). However, it should be mentioned that small inbred population with an overly low genetic diversity might hold a surprisingly high variability at the MHC complex. This is, for instance, the case of the Marsican brown bear (*Ursus arctos marsicanus*), in which a surprisingly high MHC diversity might explain its extraordinary resilience (Benazzo et al., 2017).

4.2. Demographic and biogeographic inferences

The global population size of Darwin's fox remains uncertain. Recent estimates suggest fewer than 100 mature individuals in the Nahuelbuta (Silva-Rodríguez et al., 2016) and fewer than 500 on Chiloé Island, a figure comparable to estimates for the Valdivian coastal range (Vilà et al., 2004; Farias et al., 2014) and Gorbea (D'Elia et al., 2013). Despite extensive capture efforts in the VCR and Oncol, only 3 and 2 individuals were captured, respectively. At the time of the study, no new sites of presence were detected between Nahuelbuta and Chiloé, based on 8309 camera-trap days, compared with previously known sites in the same VCR area (Farias et al., 2014). However, new records of the species have been reported in Maullín, where continuous native forest extends north VCR, based on camera trapping (Silva-Rodríguez et al., 2018). More recently (2019–2020), additional records were obtained in Mahuidanche (near Gorbea) and along the northern Sarao mountain range (Osorno coast) as part of an ongoing monitoring project (MMA LICITACIÓN 608897-86-LE19), which involved 4764 camera-trap days (Cabello et al., 2023). Finally, this elusive carnivore has also been recorded along the southern Sarao mountain range, near Los Muermos village (Balcázar, 2024). These observations suggest that the species may occur in additional localities along coastal Chile beyond those predicted by modeling (Escobar et al., 2018), albeit at very low population densities.

Coalescent analyses suggest that Darwin's fox colonized Chiloé Island during the last interglacial period, between the Last Austral Patagonian Glaciation (~180,000 years ago) and the Last Glacial Maximum (LGM; ~18,000–35,000 years ago), when the island was still connected to the mainland but partly free of ice. During the subsequent ice-sheet advance, foxes—like other species (Heusser, 1972, 2003; Villagrán, 1995; Sérsic et al., 2011)—likely found refuge in the northwestern part of Chiloé, until the ice retreated and allowed recolonization of the entire island. The presence of a unique haplotype therein is indicative of a small population size, as our time-calibrated phylogeny suggests that the population is not young (mean divergence ~140,000 years ago). The dating also indicates that the island population has remained isolated since the land bridge connections disappeared during the late Pleistocene, including the Last Glacial Maximum (LGM) and the MIS-4 period, roughly 60–70,000 years ago (García, 2012). Accordingly, our MD results reflect a post-glacial or Late Pleistocene expansion, consistent with the demographic shifts experienced by most mammals during this period, including Old (McDevitt et al., 2022) and New World canids (Chavez et al., 2022). Notably, similar patterns have been reported for several co-distributed taxa, such as the pudu deer (*Puda pudu*) (Colihueque et al., 2022), the puma (Matte et al., 2013), the Patagonian otter (*Lontra provocax*) (Vianna et al., 2004), and the iconic huemul (*Hippocamelus bisulcus*) (Marín et al., 2013).

Likewise, this scenario is consistent with the steady and ongoing decline demographic decline experienced by Darwin's fox over the past 50,000 years (Chavez et al., 2022). However, although associated with non-significant *p*-values—thus not rejecting this scenario—other MD metrics (i.e. SSD and the raggedness index) may indicate a misleading signal resulting from long-term population stability or substructure, as well as multiple expansion waves or non-neutral evolution (Rogers and Harpending, 1992). In this respect, it is worth mentioning that the ancestral *Lycalopex* lineage is thought to have dispersed westward across the Andes from present-day Argentina only about 1 mya, with the mountain range strongly constraining its early diversification (Chavez et al., 2022). Based on these considerations, a long-term stability of an ancestral population originating from a small number of founders appears more plausible than multiple colonization events.

4.3. Conservation implications

Given its low and declining population sizes (Chavez et al., 2022), compounded by the absence of gene flow, Darwin's fox is expected to experience further losses of genetic diversity through genetic drift coupled with an increasing inbreeding rate. The presence of identical MHC polymorphisms in both populations suggests that the species experienced a bottleneck prior to their divergence, consistent with a scenario of steady population decline over the last 50,000 years (Chavez et al., 2022). Nevertheless, depletion of overall genetic diversity may reduce individual fitness and increase susceptibility to infectious and parasitic diseases. This is particularly relevant for diseases transmitted by dogs, such as canine distemper (Hidalgo-Hermoso et al., 2020), which caused catastrophic population declines in the Santa Catalina Island fox (*U. l. catalinae*) (Timm et al., 2009), as well as by sympatric foxes, such as sarcoptic mange, which led to severe population reductions in Arctic foxes (Ploshnitsa et al., 2012) and is endemic in the other two fox species in Chile (Millán et al., 2024).

At present, conservation efforts aimed at securing the long-term persistence of Darwin's fox are largely limited to maintaining existing protected areas within its range, along with localized education and awareness programs encouraging dog vaccination to reduce pathogen transmission (MMA, 2014, 2016, 2018). A draft national RECOGE (Recovery, Conservation and Management Plan) for Darwin's fox has been developed and approved by local authorities in Chile; however, the lack of financial support for its implementation raises serious concerns for the species' conservation. However, important aspects of the evolutionary history and potential local adaptations of this small canid remain

unknown, and—as recently highlighted by Valenzuela-Turner et al. (2025)—the integration of genome-wide population information into conservation planning is essential for implementing effective strategies, including not only the maintenance but also the restoration of genetic variation. The availability of multiple genomes from each extant population would enable a deeper understanding of the species' evolutionary history and the consequences of the pronounced population bottleneck it appears to have experienced (Chavez et al., 2022). This would make it possible to forecast the potential consequences of low MHC diversity on individual fitness, particularly the effects of limited allelic richness on parasite load and disease prevalence (Radwan et al., 2010). Such information would be invaluable for anticipating challenges in captive breeding programs, as well as for understanding the high juvenile mortality and elevated frequency of sperm abnormalities observed in the species (O'Brien and Evermann, 1988), which are likely outcomes of its extremely low genetic diversity. Meanwhile, we advocate conducting surveys for additional populations using environmental (e.g. Franklin et al., 2019) and invertebrate (e.g. Drinkwater et al., 2021) DNA approaches in the remnants of the Valdivian Temperate Rainforest between Gorbea and Chiloé, as well as to the south and east of the island where native forests remain intact. The establishment of a robust ex-situ conservation program should be promoted to maintain captive stocks representing different populations, which could provide individuals for reintroductions or reinforcement of wild populations. Simultaneously, based on the results of this study, we recommend treating Chiloé and mainland foxes as separate management units (MUs; Palsbøll et al., 2007). This recommendation is supported by observed behavioral differences between Chiloé and mainland foxes, with the former being less fearful, as is commonly observed in island populations (Blumstein and Daniel, 2005). Additionally, subtle ecological and morphological differences have been reported between these populations (Sillero-Zubiri et al., 2004). We consider that the most effective management strategy would be the establishment of a genetic program with controlled breeding based on studied genetic profiles, crossing captive individuals from different populations to create an insurance population as a safeguard against potential catastrophes affecting wild populations. The island fox provides an illustrative example: through a targeted captive management program, its population recovered from the brink of extinction to nearly 4000 individuals, allowing its IUCN status to improve from “Critically Endangered” in 2008 to “Near Threatened” a few years later (Coonan et al., 2013). Meanwhile, we encourage the scientific community to take stock of the low genetic diversity of Darwin's fox and to reconsider its current IUCN status, which may warrant a cautious upgrade of its risk category from “Endangered” to “Critically Endangered.”

CRediT authorship contribution statement

Javier Cabello: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Giovanni Forcina:** Writing – review & editing, Visualization, Software, Methodology, Formal analysis. **Ezequiel Hidalgo-Hermoso:** Writing – review & editing, Investigation. **Darío Moreira-Arce:** Writing – review & editing, Investigation. **Constanza Napolitano:** Writing – review & editing, Investigation. **Javier Millán:** Writing – review & editing, Investigation. **Michael J. Jowers:** Writing – review & editing, Software, Formal analysis. **Warren E. Johnson:** Writing – review & editing, Resources, Investigation. **José A. Dávila:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethical statement

Not applicable.

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Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocon.2026.111997.

Data availability

All data used have been made available as supplementary material or submitted to public repositories in GenBank under accession numbers KM880155-KM880157, KJ866882-KJ866884 and PV954790-PV954792.

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