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Darwin's Fox: A Distinct Endangered Species in a Vanishing Habitat

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Abstract: *The temperate rain forest of Chiloé Island, Chile, is inhabited by an endemic fox (*Dusicyon fulvipes*) first described by Charles Darwin and now designated Darwin's fox. Despite morphological differences, Darwin's fox has been considered only an insular subspecies of the mainland chilla fox (*D. griseus*). This follows the assumption that the island population, with an estimated population of less than 500, has been separated from the mainland chilla fox for only about 15,000 years and may have received occasional immigrants from the mainland. Consequently, this island population has not been protected as endangered or bred in captivity. Recently, a population of Darwin's fox was discovered on the Chilean mainland 600 km north of Chiloé Island. This population exists in sympatry with chilla and possibly culpeo (*D. culpaeus*) foxes, which suggests that Darwin's fox may be reproductively isolated. To clarify the phylogenetic position of Darwin's fox, we analyzed 344 bp of mitochondrial DNA control-region sequence of the three species of Chilean foxes. Darwin's foxes from the island and mainland populations compose a monophyletic group distinct from the two other Chilean fox species. This indicates that Darwin's fox was probably an early inhabitant of central Chile, and that its present distribution on the mainland may be a relict of a once much wider distribution. Our results highlight the ability of molecular genetic techniques to uncover historical relationships masked by recent events, such as local extinctions. The "rediscovery" of Darwin's fox as a distinct species implies that greater significance should be given to the protection of this species and its unique habitat and to documenting the extent of its mainland distribution.*

El zorro de Darwin: una especie particular en peligro en un hábitat que está desapareciendo

Resumen: *Los bosques lluviosos templados de la isla de Chiloé, en Chile están habitados por un zorro endémico (*Dusicyon fulvipes*) que fue descrito por primera vez por Charles Darwin y que en la actualidad lleva el nombre de zorro de Darwin. A pesar de las diferencias morfológicas, el zorro de Darwin ha sido considerado sólo como una subespecie insular del zorro chilla (*D. griseus*) que habita en el continente. Esto se basa en la suposición de que la población de la isla, con un tamaño poblacional estimado inferior a 500 individuos, ha estado separada de los zorros chilla del continente por sólo unos 15,000 años y que podría haber recibido inmigrantes ocasionales del continente. En consecuencia, esta población insular no ha sido protegida como una especie en peligro, ni ha sido criada en cautiverio. Recientemente, una población de zorros de*

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*Darwin fue descubierta en la parte continental de Chile a unos 600 km al norte de la isla de Chiloé. Esta población existe en simpatria con los zorros chilla y posiblemente los zorros culpeo (*D. culpaeus*), lo que sugiere que los zorros de Darwin podrían estar aislados reproductivamente. A los efectos de clarificar la ubicación filogenética de los zorros de Darwin analizamos 344 pares de bases de la secuencia de la región control del ADN mitocondrial de las tres especies de zorros chilenos. Los zorros de Darwin de las poblaciones de la isla y del continente formaron un grupo monofilético distinto de las otras dos especies de zorros Chilenos. Esto indica que el zorro de Darwin fue probablemente un residente temprano de la región central de Chile y que su distribución presente en el continente sería un relictos de una distribución mucho mas amplia. Nuestros resultados resaltan la habilidad de las técnicas de genética molecular para revelar relaciones históricas que se encuentran mascaradas por eventos recientes, tales como extinciones locales. El redescubrimiento de los zorros de Darwin como una especie distinta, implica que se debe dar una mayor importancia a la protección de esta especie y de su hábitat particular, así como a la documentación de la amplitud de su distribución continental.*

Introduction

Chiloé Island, Chile (42–45°S, 74°W), is over 200 km long and about 30 km directly west of the coast of south-central Chile (Fig. 1). The island is the terminus of the Chilean coastal range. The southwestern portion of Chiloé Island is covered by a unique evergreen temperate rain forest that has a mixture of species from northern Patagonian and central Chilean coastal rain forests (Donoso 1993). The extreme precipitation (as much as several meters per annum) on western Chiloé Island results from an uplift of moist Pacific air over a coastal mountain range, and the high rainfall is combined with a temperate climate averaging 10°C (Smith-Ramirez & Armesto 1994). The rain forest covers approximately 2500 km² and is surrounded by agriculture and the drier parts of the island's interior. This forest complex is part of the evergreen native temperate forest type that is distributed in Chile along the coast from 39.5 to 47.0°S, intermixed with isolated islands of Alerce forests (Donoso 1993).

In 1834, during the voyage of the Beagle, Charles Darwin visited Chiloé Island, landing near the mouth of the San Pedro Channel at the southern boundary of the present-day national park (Fig. 1). Darwin collected a new species of fox, now commonly known as Darwin's fox (*Dusicyon fulvipes*). The description of this event is legendary among mammalogists; Darwin (1839:341) wrote:

In the evening we reached the island of S. Pedro . . . two of the officers landed to take a round of angles with the theodolite. A fox, of a kind said to be peculiar to the island, and very rare in it, and which is an undescribed species, was sitting on the rocks. He was so intently absorbed in watching their manoeuvres, that I was able, by quietly walking up behind, to knock him on the head with my geological hammer. This fox, more curious or more scientific, but less wise, than the generality of his brethren, is now mounted in the museum of the Zoological Society.

Darwin's fox is the rarest and most geographically isolated fox in South America, with probably fewer than 500 individuals in existence (Miller et al. 1983). It was

classified by the Chilean government in 1987 as vulnerable (Glade 1988), and the need to conduct research studies on it was considered by some to be urgent. It has not been widely recognized as threatened or endangered, however, because it occurs in remote habitats where population densities are difficult to assess, and it has an uncertain classification. The specimen collected by Darwin was apparently the only complete specimen (cranium and pelt) obtained for nearly a century, and fewer than five specimens now exist in museum collections (Medel et al. 1990). Early descriptions noted both pelage color and cranial character differences between Darwin's fox and mainland forms of the gray fox or chilla (*Dusicyon griseus*), and Darwin's fox was considered to be a different species (Osgood 1943; Cabrera 1958). Notably, Darwin's fox has a dark brown pelage with rufescent areas on its head, and it has shorter legs than the mainland foxes (Osgood 1943). Later morphological studies, however, concluded that Darwin's fox was an insular subspecies of the chilla (Langguth 1969; Clutton-Brock et al. 1976), and although these studies were based on only the few available specimens, summary treatments of mammalian taxonomy generally accept their conclusions (Honacki et al. 1982; Wozencraft 1993). Consequently, Darwin's fox was demoted to being one of five subspecies of *D. griseus* that inhabit the length of Chile and Argentina.

The recent history of Chiloé Island also supported the idea that indigenous foxes were unlikely to be distinct from their mainland relatives. Chiloé Island has been isolated from the mainland only since the last glaciation, about 15,000 years before present (Villagrán 1988), so little time has elapsed for differentiation to have occurred. Moreover, the channel separating the northern end of the island from the mainland (Fig. 1) is only about 6 km wide and 100 m deep and thus may have been crossed by mainland foxes recently or during periods of lower sea level. Such occasional gene flow would potentially arrest the processes of genetic differentiation. This scenario, however, does not consider more ancient opportunities for isolation and differentiation during the

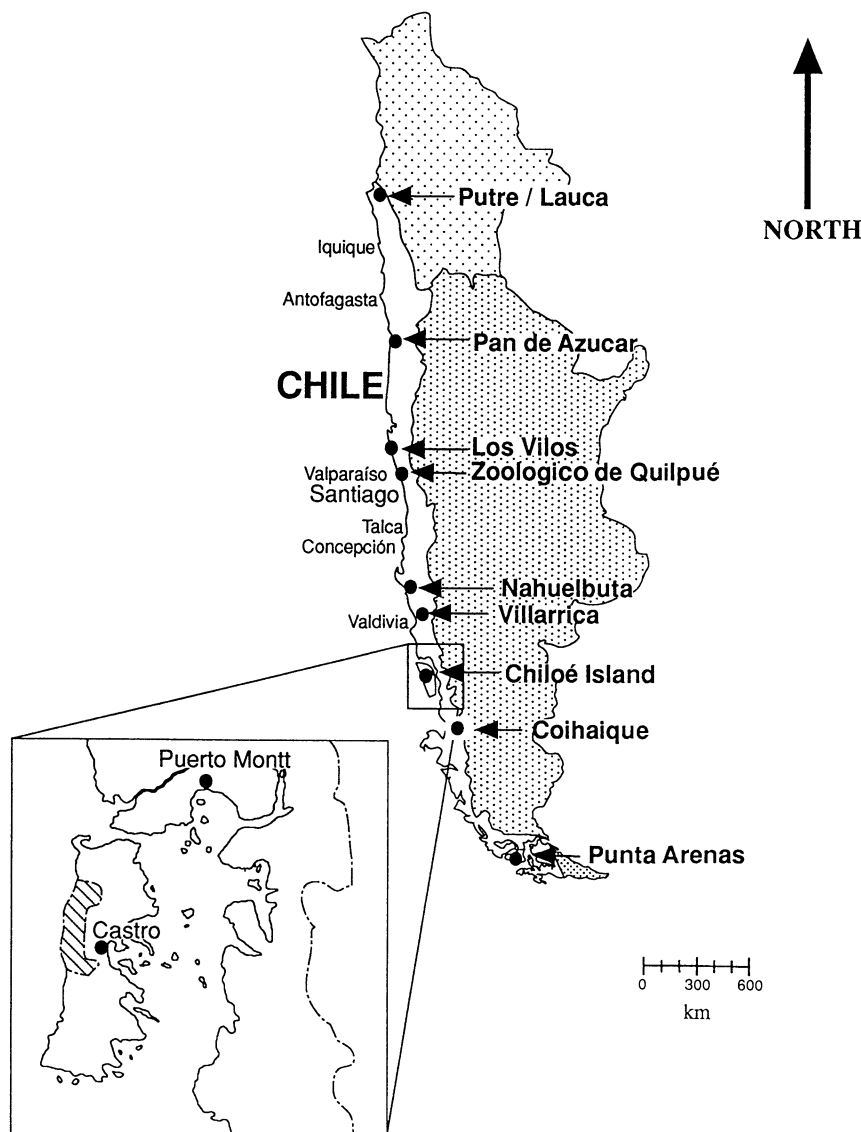


Figure 1. Localities (arrows) where fox samples were obtained and prominent cities in Chile. Striped area on Chiloé Island indicates location of the national park.

long Pleistocene history of climatic change in South America. As many as 20 glacial-interglacial cycles are thought to have occurred in the Late Pleistocene and likely effected changes in the size and degree of isolation of rain forests and forested environments (Villagrán 1988). Darwin's fox has been observed to inhabit forest and to be crepuscular, whereas the chilla fox prefers open habitat and is nocturnal (Jaksic et al. 1990; Medel et al. 1990; Jiménez et al. 1991). The changing mix of forest and open environments throughout the Pleistocene may have caused the distribution of Darwin's fox to expand in wetter periods and then contract to permanent rain forest refugia in drier periods; consequently, the species may predate the recent isolation of Chiloé Island.

Evidence for a larger historical distribution of *D. fulvipes* appeared when several individuals were discovered recently on the mainland, 600 km to the north of Chiloé Island in Nahuelbuta National Park (Medel et al. 1990). In this park (Fig. 1) Darwin's fox, chilla fox, and—at

times—culpeo fox co-exist. These three species appear to be ecologically distinct and do not interbreed. This supports the classification of Darwin's fox as a separate species and suggests a more extensive historical distribution (Jaksic et al. 1990; Jiménez et al. 1991). Conceivably, the population also could have been established with animals transplanted from Chiloé Island, although there is no evidence this occurred or that captive foxes from the island have been kept in the area (Medel et al. 1990). A further suggestion that Darwin's fox may have once had a wider distribution comes from Housse (1953), who described a chilla fox subspecies (*D. griseus fulvipes*) similar to Darwin's fox that ranged in certain forested regions of Llanquihue Province and Chiloé Island.

These hypotheses provide predictions that can be tested with molecular genetic data. If Darwin's fox is a long-isolated species, formerly with a larger historical distribution but now with a relict distribution centered

on Chiloé Island, then mainland and island populations should be genetically distinct from chilla foxes and from the much larger culpeo foxes on the mainland. Moreover, if the mainland population was only recently established with introduced animals, then they should be genetically indistinguishable from the extant island population.

We sequenced mitochondrial DNA (mtDNA) from the rapidly evolving control region to distinguish among alternative hypotheses for the origin of Darwin's fox. Mitochondrial genes are useful in evolutionary studies of mammals because they have a relatively high mutation rate, are maternally inherited, and do not undergo recombination (Brown 1986; Avise et al. 1987; Avise 1992). Furthermore, mtDNA sequence data can be analyzed using phylogenetic approaches, and hence evolutionary hypotheses can be tested. We predict that if Darwin's fox was a long-isolated species, its mtDNA genotypes should make up a monophyletic clade that is a sister taxon to mainland chilla foxes (Avise 1994). Darwin's fox on the mainland and Chiloé Island should have closely related but distinct mtDNA genotypes if they are relict populations of a species that was once more extensively distributed.

Materials and Methods

The relative taxonomic distinction of Darwin's fox was compared with that among populations of similar species on the mainland by obtaining blood samples from two fox species that likely are closely related to Darwin's fox; the chilla (2–4 kg), which is thought to be conspecific with Darwin's fox, and the larger mainland culpeo fox (5–10 kg), which is morphologically and genetically very similar to the chilla fox (Langguth 1969, 1975; Berta 1987; Wayne et al. 1989). We obtained 14 samples of chilla fox and 6 samples of culpeo fox from widely separated populations of both species (Fig. 1, Table 1). Two samples of Darwin's fox were obtained from the mainland at Nahuelbuta National Park and two sam-

ples from Chiloé Island. All individuals were measured, described, and photographed to ensure accurate species identification. Blood samples were obtained from live animals, and the animals were released unharmed.

DNA from blood samples was isolated by cell lysis followed by organic solvent purification (Sambrook et al. 1989). We determined the nucleotide sequence of 344 base pairs (bp) of DNA from a slightly larger (394 bp) fragment of the mitochondrial control region 1 amplified by the polymerase chain reaction (PCR; Mullis & Faloona 1987). Primer sets for this region were based on universal primers used in studies of vertebrates and include L15905 (5'-TAATACACCAGTCTTGTAAC-3') and H16517 (5'-CCTGAAGTAGGAACCAGA-3') (Anderson et al. 1981; Kocher et al. 1989). Each PCR reaction mixture contained approximately 100 ng of genomic DNA. DNA amplification was performed in 50 µl reactions containing 50mM KCl, 10mM Tris-HCl (pH 8.8), 0.1% Triton X-100, 2.0mM MgCl₂, 1mM dNTP mix, 2.5 units of *Thermus aquaticus* DNA polymerase (Promega), and 25 pmol of each primer. PCR amplification profiles were performed in a programmable heating block (Perkin-Elmer Cetus 9600) for 30 amplification cycles, with denaturation at 94°C for 30 seconds, annealing at 50°C for 35 seconds, and extension at 72°C for 45 seconds. Double-stranded reaction products were fractionated by electrophoresis using 2% Nusieve agarose (FMC Corp.). The appropriate sized band was excised by means of a sterile surgical blade, purified by GeneClean (BIO 101), and used as a double-stranded template for primer extension and dideoxynucleotide chain termination sequencing.

To analyze the sequence data we utilized an unweighted maximum-parsimony approach. Our outgroup sequences were from the sechura fox (*D. sechurae*) that inhabits the coastal zones of northwestern Peru and southwestern Ecuador and from the hoary fox (*D. vetulus*) that inhabits southern Brazil (Sheldon 1992). The ancestor of Chilean foxes is likely to have emigrated from the north after the emergence of the Panamanian Isthmus allowed canids to invade from North America

Table 1. Geographical distribution of mtDNA genotypes in Chilean foxes.

| Location | <i>Darwin's fox</i> (<i>D. fulvipes</i>) | | | <i>Chilla fox</i> (<i>D. griseus</i>) | | | | | | | <i>Culpeo fox</i> (<i>D. culpaeus</i>) | | | | |
|--------------|---|---------------|---------------|--|--------------|--------------|--------------|--------------|--------------|--------------|---|--------------|--------------|--------------|--------------|
| | <i>Dftu-1</i> | <i>Dftu-2</i> | <i>Dftu-3</i> | <i>Dgr-1</i> | <i>Dgr-2</i> | <i>Dgr-3</i> | <i>Dgr-4</i> | <i>Dgr-5</i> | <i>Dgr-6</i> | <i>Dgr-7</i> | <i>Dcu-1</i> | <i>Dcu-2</i> | <i>Dcu-3</i> | <i>Dcu-4</i> | <i>Dcu-5</i> |
| Putre | | | | | | | | | | | | | | 1 | 1 |
| P. Azucar | | | | | | | 1 | | | | 1 | | | | |
| Los Vilos | | | | | 1 | | | 1 | | | | | | | |
| El Soldado | | | | 1 | 1 | | | | | | | | | | |
| Z. Quilpué | | | | | | 1 | | | 1 | | | 2 | | | |
| Nahuelbuta | 1 | 1 | | | | | 1 | | 1 | | | | | | |
| Villarrica | | | | 1 | | | | | | | | | | | |
| Conguillío | | | | | | | | 1 | 1 | | | | | | |
| Chiloé | | | 2 | | | | | | | | | | | | |
| Punta Arenas | | | | | | | | | | 2 | | | 1 | | |

(Langguth 1975). We used the branch-and-bound algorithm of PAUP (Phylogenetic Analysis Using Parsimony) version 3.1.1 for the Apple Macintosh to determine the most parsimonious tree (Swofford 1993). We judged the degree of support for each node by assessing the frequency of nodes supported in 1000 bootstrap resamplings of our sequence data (Felsenstein 1985). In addition, we determined the significance of a phylogenetic signal in the sequence data by calculating the *g*1 statistic describing the distribution of 1000 random trees (Hillis & Huelsenbeck 1992).

As alternative phylogenetic approaches, we used the maximum likelihood program (DNAML) of PHYLIP modified for the Apple Macintosh computer (Felsenstein 1990). This analysis attempts to identify the tree that has the highest likelihood of yielding the sequence data, given a probabilistic model of sequence evolution. We used the empirically determined frequencies of nucleotides and an average transition-transversion ratio determined by pairwise comparisons of all taxa. As a third approach, genetic distances between genotypes were estimated by assuming a gamma distribution of rates across nucleotide sites (Tamura & Nei 1993; Wakeley 1993). A value of $\alpha = 0.5$ for the gamma distribution parameter in the Tamura-Nei model (1993) was used. This value is appropriate for sequence in control region 1 (Kumar et al. 1993; Wakeley 1993). Gamma distances, with complete deletion of missing data, were then used to construct a neighbor-joining tree, and bootstrap values were calculated using the computer program MEGA (Saitou & Nei 1987; Kumar et al. 1993).

Results

The two mainland Darwin's foxes each had a unique control-region genotype, one unique genotype was found in the two Chiloé island Darwin's foxes, seven genotypes were found in 14 chilla foxes and five genotypes were found in six culpeo foxes (Table 1). Although sample sizes were small, chilla fox genotypes had wide distributions. For example, genotypes Dgr-4 and Dgr-5 (Table 1) were found at localities separated by over 1000 km (Fig. 1). Only the two individuals from Punta Arenas and an individual from Zoológico de Quilpué had genotypes not found elsewhere. Consequently, the chilla fox seems not to be strongly subdivided with regard to its mtDNA genotypes. Individuals from each of the four localities where the culpeo fox was sampled had different genotypes, but sample sizes were too small to imply that the culpeo fox is more subdivided than the chilla fox.

Only one base-pair difference (0.4% of base pairs) separated the two Darwin's fox genotypes at Nahuelbuta, but five (1.7%) and six (2.1%) substitutions distinguished these genotypes from that found in the two individuals from Chiloé Island (Dfu-3, Table 2). Within chilla foxes the number of sequence differences between control-region genotypes varied from 1 to 11 (0.0 to 3.5%), and within culpeo foxes the number of sequence differences between control-region genotypes varied from 2 to 16 (0.8 to 7.3%). Surprisingly, the number of sequence differences between control-region genotypes of the chilla and culpeo fox, two distinct species, was sometimes

Table 2. Percentage of sequence divergence according to a gamma distribution model (above diagonal) and number of substitutions (below diagonal) for five species of South American foxes.

| | <i>Darwin's fox</i> (<i>D. fulvipes</i>) | | | | | <i>Chilla fox</i> (<i>D. griseus</i>) | | | | | <i>Culpeo fox</i> (<i>D. culpaeus</i>) | | | | | <i>D. vetulus</i> | <i>D. sechurae</i> |
|--------|---|-------|--------|-------|-------|--|-------|-------|-------|-------|---|-------|-------|-------|-------|-------------------|--------------------|
| | Dfu-1 | Dfu-2 | Dfu-3* | Dgr-1 | Dgr-2 | Dgr-3 | Dgr-4 | Dgr-5 | Dgr-6 | Dgr-7 | Dcu-1 | Dcu-2 | Dcu-3 | Dcu-4 | Dcu-5 | Dve | Dse |
| Dfu-1 | — | 0.39 | 2.06 | 5.60 | 6.07 | 5.49 | 7.17 | 6.57 | 6.57 | 6.67 | 7.16 | 5.99 | 6.53 | 5.38 | 6.46 | 10.65 | 6.53 |
| Dfu-2 | 1 | — | 1.65 | 5.14 | 5.60 | 5.03 | 6.69 | 6.10 | 6.10 | 6.20 | 6.68 | 5.53 | 6.06 | 4.92 | 5.99 | 10.11 | 6.06 |
| Dfu-3* | 6 | 5 | — | 4.03 | 4.49 | 3.97 | 5.53 | 4.99 | 4.99 | 5.03 | 5.49 | 4.46 | 5.00 | 4.92 | 5.99 | 8.98 | 5.03 |
| Dgr-1 | 12 | 11 | 10 | — | 0.40 | 0.80 | 3.01 | 3.51 | 3.51 | 2.58 | 2.52 | 3.04 | 2.55 | 5.02 | 6.06 | 9.51 | 6.81 |
| Dgr-2 | 13 | 13 | 12 | 2 | — | 0.40 | 2.58 | 3.07 | 3.07 | 3.01 | 2.96 | 2.55 | 2.09 | 5.49 | 5.59 | 8.96 | 6.35 |
| Dgr-3 | 12 | 12 | 11 | 3 | 1 | — | 2.11 | 2.58 | 2.58 | 2.54 | 2.50 | 2.11 | 1.65 | 4.94 | 5.02 | 9.67 | 5.73 |
| Dgr-4 | 16 | 15 | 14 | 8 | 8 | 7 | — | 0.40 | 0.40 | 0.40 | 3.91 | 2.61 | 2.11 | 6.57 | 6.67 | 10.22 | 6.19 |
| Dgr-5 | 17 | 15 | 13 | 10 | 11 | 10 | 3 | — | 0.00 | 0.80 | 4.41 | 3.04 | 2.55 | 5.99 | 6.07 | 9.51 | 6.82 |
| Dgr-6 | 16 | 15 | 12 | 10 | 9 | 8 | 2 | 1 | — | 0.80 | 4.41 | 3.04 | 2.55 | 5.99 | 6.07 | 9.51 | 6.82 |
| Dgr-7 | 15 | 14 | 13 | 7 | 9 | 8 | 1 | 4 | 3 | — | 3.44 | 3.14 | 2.61 | 6.06 | 7.17 | 10.80 | 6.69 |
| Dcu-1 | 15 | 15 | 14 | 7 | 7 | 6 | 11 | 13 | 12 | 10 | — | 2.97 | 3.48 | 6.21 | 7.34 | 11.04 | 7.17 |
| Dcu-2 | 13 | 13 | 12 | 8 | 6 | 5 | 8 | 11 | 9 | 9 | 7 | — | 1.30 | 6.56 | 6.53 | 10.80 | 6.05 |
| Dcu-3 | 14 | 14 | 13 | 7 | 5 | 4 | 7 | 10 | 8 | 8 | 8 | 3 | — | 5.98 | 5.99 | 10.81 | 5.53 |
| Dcu-4 | 12 | 11 | 12 | 11 | 13 | 12 | 15 | 15 | 15 | 14 | 14 | 15 | 14 | — | 0.80 | 10.23 | 7.72 |
| Dcu-5 | 14 | 13 | 14 | 13 | 13 | 12 | 15 | 15 | 15 | 16 | 16 | 15 | 14 | 2 | — | 10.31 | 7.81 |
| Dve | 22 | 22 | 20 | 21 | 19 | 20 | 22 | 20 | 19 | 23 | 22 | 22 | 22 | 22 | 22 | — | 7.98 |
| Dse | 16 | 15 | 13 | 17 | 17 | 16 | 17 | 18 | 17 | 18 | 19 | 17 | 16 | 19 | 19 | 19 | — |

*Dfu-3 is the Darwin's fox genotype found on Chiloé Island.

smaller than that observed within each species. For example, Dcu-3 was only 4 substitutions different from Dgr-3 but was 14 substitutions different from Dcu-4. Similarly, Dgr-3 differed by 10 substitutions from Dgr-5 but was only 5 substitutions different from Dcu-2. Such conflicts in sequence divergence between and within species would not be expected if both species represent monophyletic groupings (Avice 1994). In contrast, the difference between Darwin's fox and chilla fox genotypes ranged from 10 and 17 substitutions (4.0–7.2%) and was substantially greater than that within each species. Finally, the sequence difference between the two outgroup species and Chilean foxes generally exceeded that within or between the Chilean species, ranging from 13 to 23 substitutions (5.0–10.8%).

Maximum parsimony analyses resulted in a single most-parsimonious tree that was topologically similar to the neighbor-joining tree (Fig. 2) and the maximum likelihood tree (not shown). In these trees, the genotypes of Darwin's fox composed a monophyletic group that did not include genotypes from the two mainland fox species. This Darwin's fox clade was supported in 64% and 87% of bootstrap simulations in the parsimony and

neighbor-joining analyses, respectively. However, the chilla and culpeo fox control-region genotypes did not define separate monophyletic groups. Two groupings were apparent, one with a well-supported clade containing just the two genotypes (Dcu-4 and Dcu-5) found in Putre, in the northern Altiplano of Chile, and one containing culpeo genotypes from central Chile and chilla genotypes from populations ranging from southern Patagonia to northcentral Chile (Table 1, Fig. 1). Within this heterogeneous clade no strong association between phylogenetic affinity and geographic proximity was apparent. The grouping defined by Dgr-4 through Dgr-7 was comprised of genotypes from individuals from diverse localities ranging from northcentral Chile (Los Vilos) to southern Patagonia (Punta Arenas). But the individuals of the clade defined by Dgr-1 through Dgr-3 are all from central Chile.

Discussion

Analysis of 344 bp of the control-region sequence demonstrated that Darwin's fox is not an insular subspecies

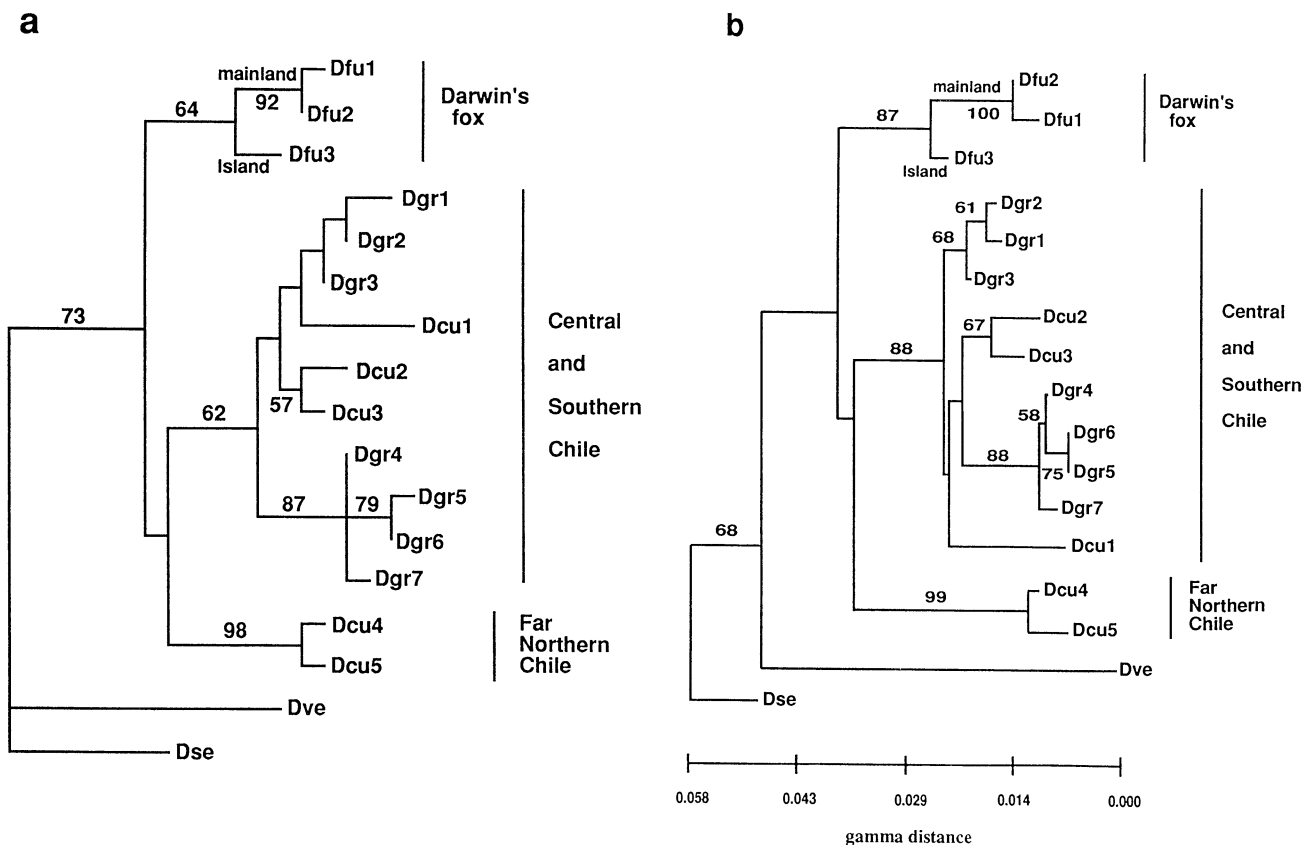


Figure 2. Two phylogenetic trees showing the relationship between control-region genotypes of island and mainland foxes: The most parsimonious phylogenetic tree (tree length = 71, overall consistency index = 0.63, $g1 = -0.73$) (a) and the tree generated by the neighbor-joining method (b). Nodes supported in more than 50% of 1000 bootstrap resamplings of the sequence data are indicated before bifurcations on the phylogenies.

of the mainland chilla fox but rather is a distinct species, as Darwin originally surmised. The average sequence divergence between Darwin's fox genotypes and those of the chilla fox was about $5.6 \pm 0.89\%$ (range 4.0–7.2%). In contrast, the sequence divergence among chilla genotypes from localities separated by over 2000 km ranged from 0.4 to 3.5% (Table 2). Darwin's foxes formed a monophyletic clade that was a sister taxon to the clade containing chilla and culpeo fox genotypes. This suggests that Darwin's fox is perhaps an evolutionary relict of a more ancient invasion of foxes into South America. The estimated divergence time between Darwin's fox and the chilla fox is about 275,000 to 667,000 years ago, assuming a rate of divergence in control-region sequences varying from 8.4 to 20.4% per million years (Vigilant et al. 1989, 1991; Wenink et al. 1993). The chilla fox is not represented in the fossil record, but the culpeo, hoary, and sechurae fox are known from the Late Pleistocene Lujanian period, which spans a period of 300,000 to 10,000 years before present (Webb 1985; Berta 1987, 1988). Therefore, our results suggest that Darwin's fox is a distinct species that originated from a Late Pleistocene invasion of foxes into South America.

The mainland population of Darwin's fox may be either a remnant of a once much-more-widely distributed species or recently established by transplantation of animals from Chiloé Island. There are no records of animals being released from Chiloé Island onto the mainland, however, and the only individual known with certainty to have been kept in captivity died in the Santiago zoo in 1977 (Medel et al. 1990). Our results show that the two genotypes found on the mainland are very similar to each other but differ by about 2% in DNA sequence from a genotype found in two Chiloé Island foxes. This degree of sequence divergence is consistent with that expected among widely separated and long-isolated populations, but not with that found within a single, small population. Theoretical considerations suggest that in small, isolated populations mtDNA genotypes should coalesce to a single common ancestor in approximately $4N_f$ generations, where N_f is the female effective population size (Avise et al. 1988). Given a generous female effective population size of a few hundred individuals and a generation time of 2 years, the coalescence of genotypes should be on the order of a few thousand years. Consequently, given even higher rates of sequence evolution, the amount of divergence between island genotypes would not be expected to exceed 0.5%.

In addition, small populations are unlikely to have many control-region genotypes. Our results predict that if the mainland Nahuelbuta population had been established from captive animals, at a minimum two females must have founded the population, because we found two genotypes there. Thus, three genotypes must exist within the island population. The expected number of genotypes in a nonsubdivided, freely-interbreeding popu-

lation may be related to population size at equilibrium as $n_e = 2N_f\mu + 1$, where N_f is the effective female population size and μ is the mutation rate (Birky et al. 1983, 1989). For a population of less than a few hundred females and mutation rates of less than 10^{-5} per generation per site, only one or two genotypes are predicted to exist (Lehman and Wayne 1991; Wayne et al. 1992; Gottelli et al. 1994). Even given the highest cited mutation rate for control-region sequences, about 10^{-6} per generation per site (Ward et al. 1991; Lundstrom et al. 1992), it seems unlikely that three genotypes existed within the island population.

Empirical studies of small populations of canids support these predictions. Genetic studies of populations of California island fox (*Urocyon littoralis*) have found that island populations of a few hundred females or less have only one or two mtDNA restriction-site genotypes (Wayne et al. 1991). A more comparable study surveyed control-region sequences of 49 individuals from an isolated population of fewer than 500 Ethiopian wolves and found only one mtDNA genotype (Gottelli et al. 1994). Therefore, the presence of three distinct genotypes in our sample of four Darwin's foxes is more consistent with the hypothesis that the mainland Nahuelbuta population is a relict of a once more-widely dispersed species. This hypothesis needs to be tested with additional samples from the island, and efforts should be made to survey other mainland areas for Darwin's foxes.

Chilla and culpeo fox genotypes did not define separate monophyletic clades if, as expected, the two species were reproductively isolated and had long, distinct evolutionary histories. Given the low values of sequence divergence between the two species, however, they may have diverged recently, and thus their mtDNA genomes may not have had sufficient time to reach reciprocal monophyly (Avise et al. 1984; Avise 1994). The mitochondrial genotypes of two recently diverged species are expected to have first polyphyletic, then paraphyletic, and finally monophyletic relationships (Avise 1994). Reciprocal monophyly is not expected until about $4N_f$ generations (Avise et al. 1988; Avise 1994). Because both species have a very large population, perhaps several hundred thousand individuals (Crespo & De Carlo 1963; Duran et al. 1985; Johnson & Franklin 1994), their genotypes may not be expected to coalesce to separate common ancestors until several hundred thousand years have passed. Thus, the observation that the mtDNA genotypes of the culpeo fox define a paraphyletic group (Fig. 2) may result from recent speciation and large population size. A similar explanation has been applied to explain the paraphyletic relation of mallard and black duck mtDNA genotypes (Avise et al. 1984). An alternative explanation is that the chilla and culpeo fox may hybridize in areas where they are sympatric, thus exchanging genotypes between the species (Lehman & Wayne 1991;

Mecure et al. 1993). But the observation that no genotypes are identical in the two species (Table 1) argues against hybridization. More-extensive sampling of both species across their geographic ranges and information from additional mitochondrial regions are needed to understand better the origin of the paraphyletic pattern of culpeo and chilla fox genotypes.

Implications for Conservation

Darwin's fox appears to be more closely associated with forest and scrub habitats, whereas the chilla fox prefers open areas (Miller et al. 1983; Medel et al. 1990). Palynological data indicate that from 12,500 years ago to the present, forest plants dominated the pollen profile of central Chile (Villagrán 1988; Ashworth et al. 1991). Recent habitat changes caused by human activities have greatly reduced the area covered by forest, and open habitats now dominate (Armesto et al. 1987; Quintanilla 1987). Deforestation occurred relatively slowly from the early 1800s until the 1950s, principally to clear land for agriculture. But the pace of deforestation has increased dramatically in the last 20 years. From 1975 to 1985, 30% of the native forests on the coast of southcentral Chile (eighth region) were replaced by pine (*Pinus radiata*) plantations, and the production of chips (20% of which come from native forests) has increased from 13.9 thousand tons in 1986 to 1700 thousand tons in 1991 (CODEFF: Comité Nacional pro Defensa de la Fauna y Flora 1992). Southwestern Chiloé Island has remained the largest continuous closed-habitat rain forest in Chile, in large part because of its isolation, inaccessibility, and inclement weather. On the mainland, only smaller, noncontiguous fragments of coastal forest habitat remain, and, considering the seemingly low abundance of Darwin's foxes in forested habitats (Jiménez et al. 1991), many mainland habitats may not be sufficiently large to sustain viable populations. Nahuelbuta National Park, with only 5415 ha, represents the only known mainland location where Darwin's fox exists; it is an example of a coastal closed-forest habitat that deserves more study and more protection if this species is to be conserved. Because there are few areas of comparable size and habitat left on the mainland, and none of these is protected, Chiloé Island and Nahuelbuta National Park may remain the only viable, long-term refugia for Darwin's fox.

Clearly, forested areas along the coastal ranges that link the Cordillera de Nahuelbuta and Chiloé Island should be surveyed for the presence of Darwin's fox. The rain-forest habitat of Chiloé is unique, and its extensive area might sustain a large population of this otherwise rare species. The population of foxes that exists on the island may be close to the minimum of 500 needed to maintain long-term viability, given random demographic

fluctuations (Lacy 1987; Lande 1988). Because a single population may be vulnerable to local environmental effects or epizootics however, (O'Brien & Evermann 1988), it would be important to determine if Darwin's fox exists or has existed in other mainland forests so that these areas could be targeted for increased protection or reintroduction. Other mainland populations may exist, because the ninth region, which includes Nahuelbuta National Park and Chiloé Island, retains 48% of Chile's nonprotected native forest.

Because of the limited distribution and low population numbers of *D. fulvipes*, a captive-breeding program, preferably within the country, should be established. This would serve to provide a source for reintroduction in the event of a catastrophe within the island population and to obtain more-detailed knowledge of the disease and reproductive parameters of Darwin's fox.

Finally, the temperate rain-forest habitat of Chiloé and the coast is unique, with elements of Patagonian and central Chilean rain-forest habitats. Forty to 49% of bird species and 67% of mammal species found in the temperate Chilean rainforest are endemic, and a new rodent genus has recently been described (Vuilleumier 1985; Benoit 1989; Patterson 1992; Willson et al. 1994). Several species of plants classified as vulnerable in Chile are found in this area, including Alerce (*Fitzroya cupressoides*), which is protected in Chiloé Island National Park, and Araucaria (*Araucaria araucaria*), which is protected in Nahuelbuta National Park. Only a small portion of this unique native forest is protected, however. Only 2% of the remaining 150,000 ha of the high-altitude forest of Nahuelbuta is protected, and agroforestry threatens unprotected areas. Similarly, only 5% of the 1,150,000 ha of "Laurifolio" forest of Chiloé Island is protected, and agricultural forest exploitation threatens much of this area as well (Ortiz et al. 1993). Darwin's fox could serve as a flagship species for this habitat. By preserving the habitat, other, less charismatic but equally interesting and distinct species, such as the Chilean "shrew" opossums (*Rhyncholestes* spp.), would be protected. In addition, the highest number of tree species endemic to Chile are found between 38° and 43°S (Smith-Ramirez & Armesto 1994), the same area potentially occupied by Darwin's fox. The results of this study demonstrate the value of molecular systematic and natural history studies in uncovering the evolutionary significance and the role of endangered taxa in vanishing ecosystems (Avise & Nelson 1989; May 1990; Daugherty et al. 1990; Avise 1992).

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