

Record and distribution of black-fur foxes in a Mediterranean natural park, Serra de Mariola, Spain

Antonio Belda^{1*} & Eduardo Larriba^{2,3}

1. Environment and Earth Sciences Department, University of Alicante, Apdo 99, 03080 Alicante, Spain.
2. Multidisciplinary Institute for Environmental Studies (MIES) “Ramón Margalef”, Department of Marine Sciences and Applied Biology, University of Alicante, Apdo 99, 03080 Alicante, Spain.
3. Department of Cellular and Molecular Biology, Centro de Investigaciones Biológicas (CSIC), 28040 Madrid, Spain

Corresponding author: antonio.belda@ua.es

The red fox *Vulpes vulpes* (Linnaeus, 1758), is one of the most widely distributed mammalian species across the globe, living in a wide range of temperature zones, geographical regions, wild forest and urban environments (Macdonald & Reynolds 2004). Since the early 20th century, the red fox has also become a common fur-farm animal in North America and Eurasia (Statham et al. 2011). Fur farmers industries have raised two color variations in common red foxes: the ‘silver’ fox with a melanic variation (black-fur), and the ‘cross’ fox with a reddish fur color but exhibiting a darker line along the back of the animal, being the specimens ‘silver’ and ‘cross’ unusual in Europe (López-Martín 2010). Non-native fox subspecies can hybridize with closely-related native species or subspecies, potentially leading to loss of locally adapted alleles, disruption of co-adapted gene complexes, other forms of outbreeding depression, or extinction via genetic swamping (Sacks et al. 2011, Canu et al. 2014). Melanic fox were documented in Mediterranean environments in Spain (Serra del Tallat, Lleida; Parc del Garraf, Barcelona; Paraje Natural Peña Escrita, Granada; Parque Natural dels Ports- Tarragona, personal communications). The aim of this work is to show the presence of foxes with dark fur and see the relationship with the common fox, using camera-trapping methods and mitochondrial genetic markers, in a protected natural area in Mediterranean environments.

The Serra de Mariola Natural Park is located in the Southeast region of Spain, in the north of Alicante province and south of the Valencia province (Fig. 1). The total area occupied by the protected land spans 17,500 ha. This area exhibits a highly mountainous and rugged relief, except for some river valleys. Due to its geographical location, Serra

de Mariola has a typical Mediterranean climate with mild temperatures. According to the land uses, the most abundant is the forest matrix, which occupies 67% of the total area of the park, followed by the dry (24%), urban (5%), abandonment (3%) and irrigation (1%) matrices. This Natural Park harbors a high degree of plant and animal biodiversity (Belda et al. 2013a, 2013b).

During the years 2009-2011 we studied the influence of landscape structure on the red fox community in the Serra de Mariola Natural Park. The study area was divided into 63 cells of 4 km² each, and two camera-traps, separated by a minimum distance of 200 m were installed in each cell for observation period of 15 days (Zaragozi et al. 2015). Moreover, hair samples from six fox specimens were collected, using hair traps, from six different sampling sites (Fig. 1). The cameras filmed the trapping site, so that we could document the fur colour of the trapped animals (three with red- and three black-fur individuals). Hair samples, collected with field traps, were used for DNA isolation using the DNA easy blood and tissue kit (Qiagen). Haplotype determination was based on the mitochondrial DNA-encoded cytochrome b gene (CYTB, GeneID: 435576), according to Frati et al. (1998). This gene was PCR-amplified from total DNA isolated from hair samples and the amplified fragment obtained was subjected to automatic DNA sequencing. The sequences obtained were aligned using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and deposited in the GenBank database under accession numbers: KM225298 to KM225303. A Bayesian consensus tree of the sequences was constructed using MrBayes (Huelsenbeck & Ronquist 2001). The HKY model with gamma-distributed rate

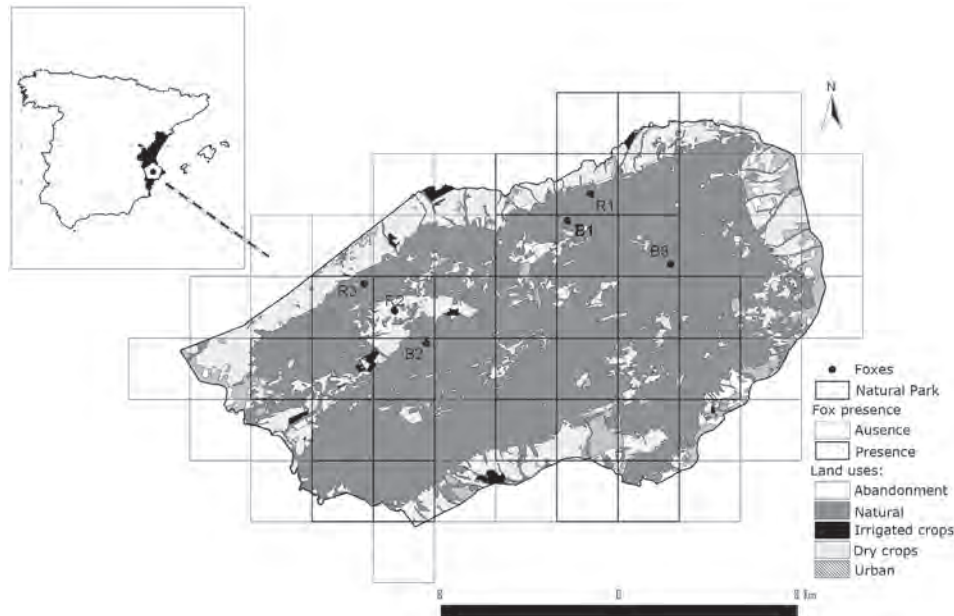


Figure 1. Land uses in the Serra de Mariola Natural Park showing the distribution of red foxes. Points indicate the sites of sampling for DNA analysis (R1-R3, red-fur foxes; B1-B3, black-fur foxes).

variation across sites was used for phylogenetic reconstruction. Sampling was set to every 100 generations for 3×10^6 generations with four chains (average standard deviation of split frequencies < 0.02). After discarding burn-in trees, the remaining samples were retained for generating consensus trees, calculating 95% Bayesian credibility intervals and posterior probabilities.

Our studies based in the landscape distribution of red fox individuals showed that foxes in the Serra de Mariola Park were present in 49.2% of sampling cells. The red fox was widely distributed throughout the Natural Park in natural areas with dry and irrigated crops, avoiding both peripheral and anthropic areas (Fig. 1). The camera-trapping study allowed us to detect the presence of red- and black-fur (Fig. 2) foxes occupying the same areas and possibly the same home-range, representing the first genetic characterization of black-fur foxes residing in Mediterranean forests. In order to understand the population relationship between individuals, bearing the two fur types we performed a MT-CYTB gene analysis. This analysis revealed the presence of two distinct alleles in the population, which differed in a single nucleotide polymorphism (SNP) in position 171 of the open reading frame, where a nucleotide transition (C-T) had occurred. The allele 171C was found to be present in both black- (B1-B3) and red-fur (R3) individuals, and the other allele (R1, R2) found in the Serra de Mariola

red fox. Phylogenetic analysis of cytochrome b sequences (Fig. 3) showed that sequences from allele 171C form a single cluster that groups in the clade II (Fig. 3), suggesting that these sequences present a relationship with these haplotypes. Clade II groups different sequences from European foxes, including the haplotype 171T sequences (Fig. 3). Haplotype 171T corresponds to that previously described in other Iberian Peninsula fox populations (Fernandes *et al.* 2008).

Camera-trapping represents a fundamental technique for the study and conservation of mammals in natural environments (Rovero *et al.* 2013). This method allowed us to detect the presence of black-fur foxes intermingled with the local red-fur fox



Figure 2. Image of black-fur fox individual.

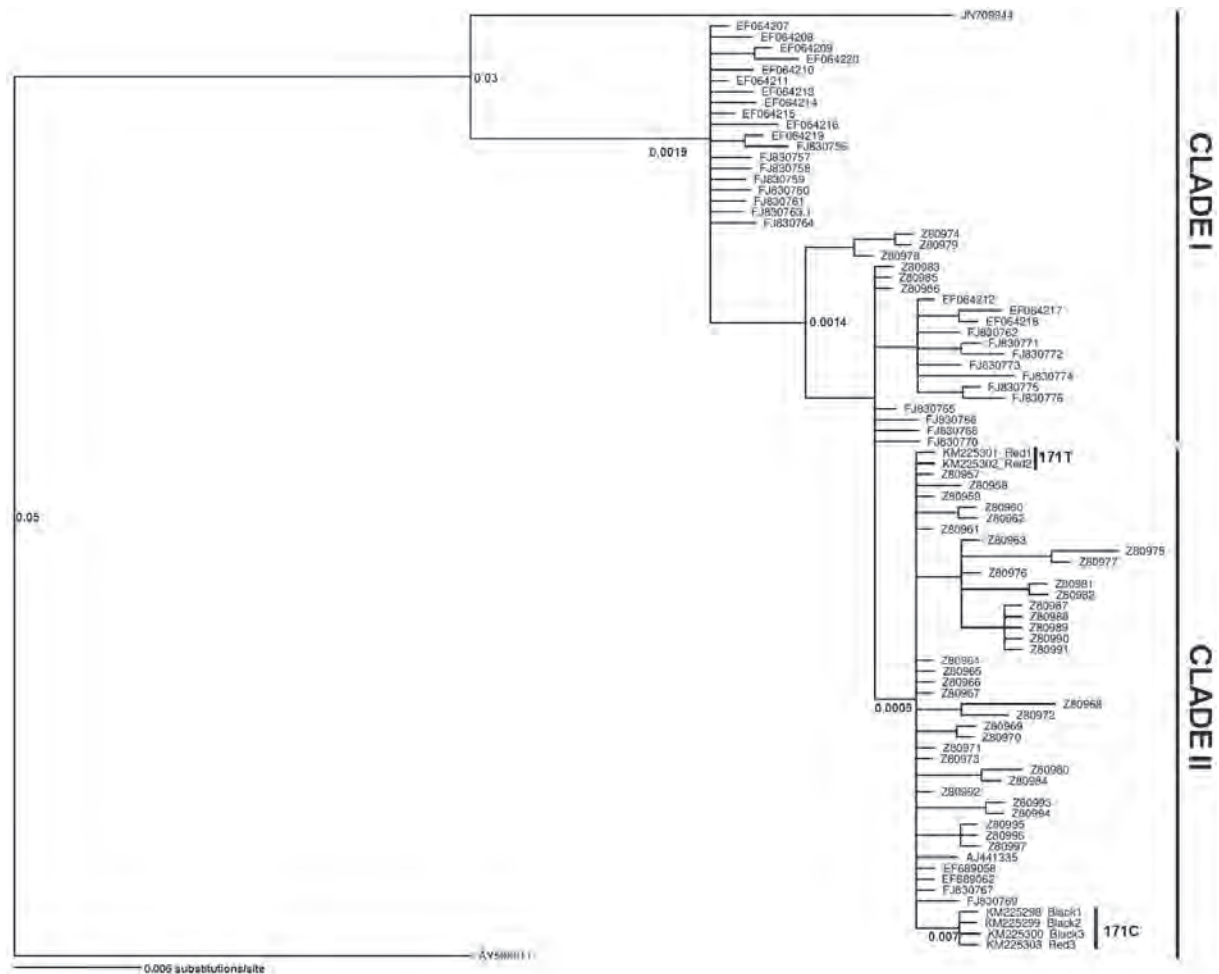


Figure 3. Bayesian tree produced using a dataset including cytochrome b sequences from Frati *et al.* (1998) [Z80957-Z80997], Perrine *et al.* (2007) [EF064207-EF064220], Palomares *et al.* (2002) [AJ441335], Fernandes *et al.* (2008) [EF689058, EF689062], Aubry *et al.* (2009) [FJ830756-FJ830776] and unpublished sequence JN709944 (Dang *et al.* 2011). This analysis also included the sequences of these study, haplotype 171T (R1, R2) and haplotype 171C (B1-B3, R3) [KM225298-KM225303]. Cytochrome b sequence from *Alopex lagopus* (AY598511) has been used as outgroup. Clade I corresponds to North American and Euroasian regions. Clade II corresponds to European region. Numbers adjacent to the node are Bayesian posterior probabilities.

populations. To examine the characteristics of the fox population, a haplotype analysis was performed based on the mitochondrial DNA cytochrome b gene (MT-CYTB), which has been widely used for the characterization of fox populations (Statham *et al.* 2011, Edwards *et al.* 2012, Ibiş *et al.* 2014). This study allowed us to identify a new CYTB allele, which suggest the hypothesis of a mixture of black-fur foxes coexisting with individuals of the original population in Serra de Mariola. In addition, this is the first study about fox haplotypes carried out in Serra de Mariola Natural Park, which constitutes a typical Mediterranean ecosystem (Belda *et al.* 2013b). Our genetic studies on populations of foxes in Serra de Mariola opens the door to the possibility of hybridization between native red-fur foxes, and

immigrant black-fur individuals. Further studies based on autosomal markers and specific color associated genes (Kukekova *et al.* 2012) are needed to understand the origin of these new black-fur individuals. This phenomenon would be favored by a lack of competition between the two phenotypes for food resources, given the high density of wild rabbits existing in this area (Arques *et al.* 2010). Genetic studies are necessary for the conservation of natural populations and the study of their interactions with non-native subspecies (Sacks *et al.* 2011), and impact on natural communities in many different ways, including the introduction of novel pathogens, reduction of native prey populations and competition with native species for food resources (Mack *et al.* 2000, Manchester

& Bullock 2000, Canu *et al.* 2014). Future studies on fox densities and gene flow taken place within genetic population structure of Serra de Mariola, are needed to understand the presence of black-fur foxes and reduce their environmental impact.

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