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Short communication

Hybridization between wolf and domestic dog: First evidence from an endangered population in central Portugal

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ABSTRACT

Human population expansion has promoted contact between wildlife and domestic animals with severe ecological consequences, such as anthropogenic hybridization. In Portugal, Iberian wolf (*Canis lupus signatus*) populations are considered "Endangered" and co-habit with humans so the risks of hybridization with free-ranging dogs, and livestock depredation can be particularly high. Our aim was to report the occurrence of wolf-dog hybridization in an endangered Iberian wolf sub-population, located in the south of the Douro river, Portugal. We used mitochondrial DNA and microsatellite data to investigate putative hybrids between Iberian wolves and dogs. Here, we report for the first time a wolf-dog hybrid located in the south of the Douro river. This is the second hybrid found in Portugal, and even if hybridization cases are still considered rare, they can be particularly problematic in isolated, fragmented and endangered populations, such as the one studied here. Appropriate management and conservation measures are recommended.

As human population increases in numbers and expand, also into wildlife suitable habitats, the contact between wildlife and domestic animals is likely to intensify, with several ecological consequences, such as anthropogenic hybridization (Allendorf et al., 2001). Anthropogenic hybridization, triggered by human-induced changes (*e.g.*, habitat modification, fragmentation, species (re)introductions) (Allendorf et al., 2001), has prompted intense debate (Vilà and Wayne, 1999). It has been suggested that anthropogenic hybridization has several undesirable consequences, jeopardizing the genetic integrity of populations, eventually causing their extinction (Lescureux and Linnell, 2014). One of the classic examples of the potential deleterious contact between wildlife and domestic animals is the hybridization between wolf (*Canis lupus*) populations and free-ranging dogs (*Canis lupus familiaris*). Wolf-dog hybridization has long been acknowledged in Europe (Randi, 2011), though occurring with different frequencies: low frequency in the western regions (*e.g.*, Italy: Randi and Lucchini, 2002; Iberian Peninsula: Godinho et al., 2011a) and high in the eastern part of the continent (*e.g.*, Estonia, Latvia, Bulgaria: Randi et al., 2000; Andersone et al., 2002; Hindrikson et al., 2012; Caniglia et al., 2014).

Contrary to the remarkable wolf recovery, which occurred in several European countries (Chapron et al., 2014), the distribution of the Iberian wolf (*Canis lupus signatus*), an endemic subspecies of the Iberian Peninsula, has been declining throughout the 20th century in Portugal (Torres and Fonseca, 2016), where it is considered "Endangered". Several studies report that hybridization is particularly problematic in small and fragmented wolf populations, inhabiting humanized landscapes, where free-ranging dogs are common (Vilà and Wayne, 1999; Godinho et al., 2011a) and where their feeding ecology is largely based on livestock (Torres et al., 2015; Torres and Fonseca, 2016). Consequently, it is vital to identify the degree of hybridization between wolf and free-ranging dogs in endangered and small populations, such as the subpopulation presented in this study. Here, we investigate the occurrence of wolf-dog hybrids in an Iberian wolf subpopulation in central Portugal (south of the Douro river), at the south-western edge of its distribution, using mitochondrial DNA and microsatellite data.

The study was conducted in central-west Portugal, within an area of 750 km^2, which encompasses the range of 3 wolf packs (Arada, Montemuro and Cinfães packs, Fig. 1) (for more details of the study area please see Torres et al., 2015). In Portugal, genetic studies have demonstrated the existence of two apparently isolated wolf subpopulations, separated by the river Douro (Godinho

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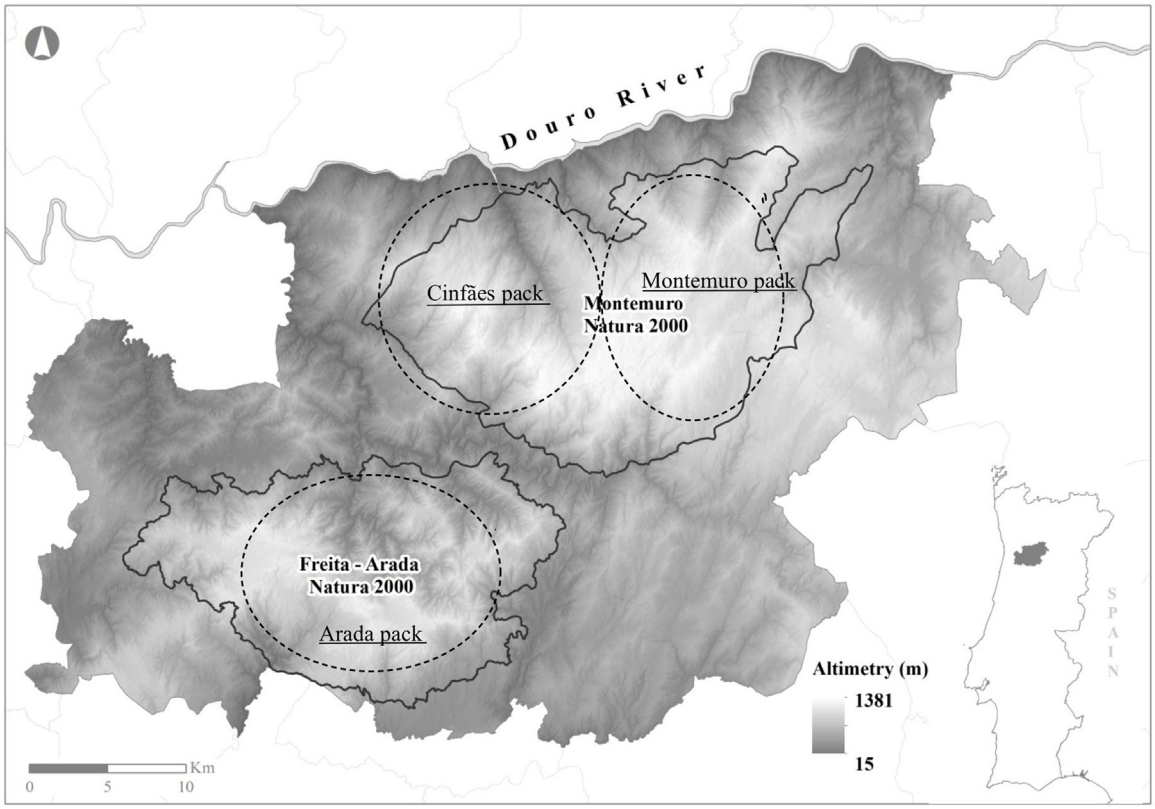

Fig. 1. Location of the study area in the south of the Douro river, highlighting the distribution of the three studied wolf packs.

et al., 2011a): the packs north of this river are considered stable and appear to be locally expanding; while the packs south of this river (only 6 confirmed packs) are isolated from the remaining populations, showing high levels of fragmentation and low genetic diversity (Godinho et al., 2011a; Hindrikson et al., 2016).

A total of 47 transects were distributed throughout the study area, and were monthly inspected, which corresponds to 130.4 km *per* month (smallest transect: 0.6 km; largest transect: 7.4 km), from 2011 to 2014. We also collected scats opportunistically while travelling to and between transects. During this period, a total of 93 scat samples were carefully collected (to avoid contamination) in the territory of the three packs and stored immediately in 96% ethanol, and after a few hours at −20 °C. During the same time period, saliva and hair samples were randomly collected from 26 domestic dogs (mainly shepherd, stray and hunting dogs) from the same area. Hair samples were also collected from a juvenile animal with a phenotypic appearance of a wolf (J0A) found dead in the territory of the Arada pack, in September 2014 (unknown cause of death). The DNA was isolated from scats, saliva or hair samples, one to two weeks after collection using the QIAGEN® QiAamp DNAStool kit, and from tissue using a standard salt-out extraction procedure (Bruford et al., 1992). All laboratory procedures were held in dedicated facilities, and with all due care in order to avoid contamination of samples. Scats, saliva and hair samples usually have low amount of DNA, with low average quality. A 442bp-length fragment of the D-loop (mtDNA) was sequenced for all samples, using the primers Thr-L 15926 and DL-H 16340 (Vilà et al., 1999). However, the comparison of mtDNA haplotypes was based on a shorter sequence (261 bp), following Vilà et al. (1999), since no polymorphism was detected outside this shorter fragment. This fragment is frequently used in the molecular distinction between wolf and dog (*e.g.*, Godinho et al., 2011a). All samples were also genotyped for a panel of 24 microsatellite markers, based on at least three replicate genotypes for each marker. This panel included the 18 markers included in the Canine Genotypes™ 1.1 (Finnzymes®) and six additional markers, that were amplified in two multiplex reactions: the first including the markers C04.140, C20.253 (Ostrander et al., 1993), FH2001 and FH2161 (Francisco et al., 1996); the second including the markers CPH14 (Fredholm and Wintero, 2009) and DBAr (Kerns et al., 2004). Mitochondrial haplotypes were compared with those from wolves and domestic dogs identified by Vilà et al. (1997), that we retrieved from Genbank (Accession numbers: AF005280.1–314.1; AF008135.1-82.1; https://www.ncbi.nlm.nih.gov/genbank/). Comparisons of mtDNA haplotypes were performed using a neighbour-joining phylogenetic tree, generated with the algorithm implemented in MEGA 6 (Tamura et al., 2013). General diversity indices, assignment tests and principal coordinate analysis (PCoA) were performed using GenAlEx 6.501 (Peakall and Smouse, 2012). For the PCoA, and subsequent NEWHYBRIDS and STRUCTURE analysis, our dataset of wolf and dog genotypes was complemented with a dataset of genotypes of Iberian wolf and domestic dog (Godinho et al., 2011a), freely available on Dryad (Godinho et al., 2011b). This dataset included 408 genotypes based on 42 autosomal microsatellite markers. After discarding the most incomplete genotypes from the dataset, we included in our analysis: 191 domestic dog genotypes, 197 wolf genotypes and 8 wolf-dog hybrids, identified in Godinho et al. (2011a). We only incorporated in our analyses the data pertaining to the 24 microsatellite markers used for genotyping our samples. In order to allow the calibration and merging of the two datasets, a reference sample (SMLM88, from the Portuguese National Tissue Collection ICNF) was used. This sample, that has been previously genotyped at Godinho's laboratory, was the first sample to be genotyped in our lab. We used this sample to calibrate the allele calling procedure and genotyped all samples using the calibrated allele calling. Additional checking was performed by carefully inspect-