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Short Communication

Genetic variation and subspecific status of the grey wolf (*Canis lupus*) in Saudi Arabia

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ABSTRACT

This work represents the most extensive genetic study of the grey wolf (*Canis lupus* Linnaeus, 1758) in Arabia and the first considering genetic data from multiple locations within Saudi Arabia. Previous suggestion of the occurrence of two subspecies of wolves in Arabia is not supported by this study. The genetic evidence suggests that the wolves of Saudi Arabia are genetically variable and more closely related to the Eurasian wolf *Canis lupus* group (dog included) than to the Indian wolf *C. l. pallipes*. The genetic diversity observed for *C. lupus* in Saudi Arabia indicates that the subspecific status *C. l. arabs* should be retained for the Arabian wolf. What remains unclear is the degree to which genetic introgression from domestic dogs has influenced the composition and integrity of *C. lupus* in Saudi Arabia.

The diversity of the grey wolf, and the validity and distributions of the many described subspecies, are far from resolved (Rueness et al., 2011; Gaubert et al., 2012). This is partly due to the very wide distribution of *C. lupus* as well as the interactions among the subspecies (IUCN, 2013). Evidence suggests *C. lupus* was the first animal to be domesticated by humans (Clutton-Brock, 1995) and multiple origins of domestication have been suggested, most recently in South East Asia (Pang et al., 2009) as well as both the Middle East and Europe (vonHoldt et al., 2010). The earliest dog (C. l. familiaris) remains in the archaeological record have been described from Belgium, the Middle East, and western Russia 12–31,000 years ago (Dayan, 1999; Germonpré et al., 2009). Due to the recent divergence between wild *C. lupus* and dog populations, genetic similarity is high (~99.4% identity of mitochondrial genome; Arnason et al., 2007). This is likely attributable to on-going gene flow between wild wolves and domestic dogs (Khosravi et al., 2013). It is not surprising that *C. l. familiaris* is hybridizing with its wild progenitors in an increasingly human-dominated landscape (Anderson et al., 2009), although there is a notable absence of obvious *C. l. familiaris* genetic introgression at some sites (e.g., Lithuania; Baltrunaite et al., 2013). As human (and, by association, *C. l. familiaris*) populations have increased, there are ever greater opportunities for genetic introgression eroding the diversity of wild populations of *C. lupus*, as is being seen in the wildcat (*Felis sylvestris*) (e.g., Beaumont et al., 2001).

Extensive genetic research has been conducted worldwide across populations of *C. lupus*, but difficulty in comparing studies has arisen due to the use of just one of two mitochondrial genes (rather than both); control region (CR), or cytochrome *b* (cytb), or any of a range of available nuclear microsatellites. Even where facilities exist for using microsatellites, the large numbers available mean that there are regularly no over-lapping loci among studies (e.g., Baltrunaite et al., 2013; Cohen et al., 2013; Khosravi et al., 2013; =52 non-overlapping loci).

Canis lupus is the most common large wild carnivore in Saudi Arabia; a putative figure puts the population size at >500 individuals (Mech and Boitani, 2004). There is uncertainty regarding the subspecific status of *C. lupus* in Saudi Arabia. Some sources indicate presence of the Indian wolf (*C. l. pallipes*; Sharma et al., 2004; Rueness et al., 2011) in Saudi Arabia, while others indicate

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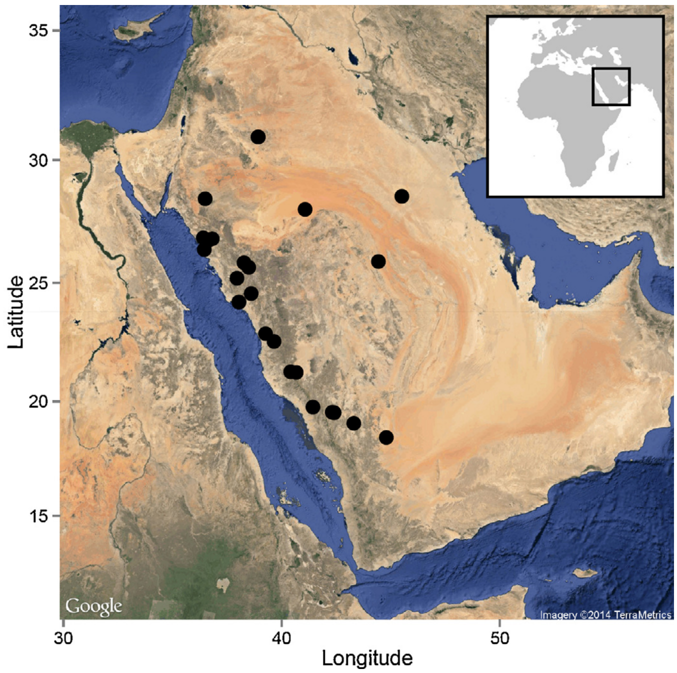

Fig. 1. Sampling locations in Saudi Arabia for which *Canis lupus* genetic data were obtained ($n = 37$; image generated in ggmap package in R).

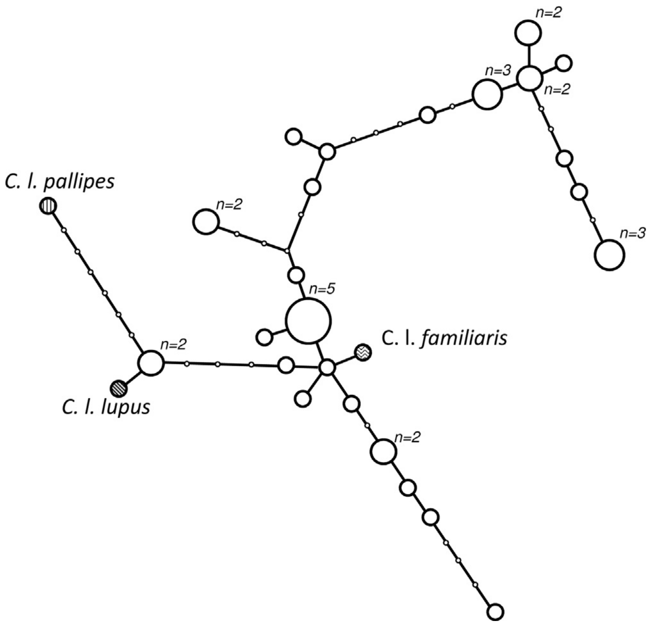

Fig. 2. Combined control region and cytb haplotype median joining network with 37 *Canis lupus* samples from Saudi Arabia and a representative sample each of *C. l. pallipes* (India), *C. l. lupus* (Sweden), *and C. l. familiaris* (Egypt) (GenBank accession numbers AY333749, NC009686, and JQ088658/JQ088677, respectively).

presence of the Eurasian wolf (*C. l. lupus*; Vilà et al., 1997). Harrison and Bates (1991) follow Pocock (1934) in assigning *C. lupus* of Saudi Arabia to yet a third subspecies, the Arabian wolf *C. l. arabs*, but some researchers do not recognize this subspecies (e.g., Mech and Boitani, 2004; Rueness et al., 2011; Gaubert et al., 2012;). This leads to the question of which subspecies of *C lupus* is/are present in Saudi Arabia at this time. To date, published *C. lupus* sequence data attributed to Saudi Arabia are limited to two studies of the same four individuals, all of unknown origin (Ellegren et al., 1996; Sharma et al., 2004).

Here we apply two of the most commonly employed mitochondrial gene regions to address the following questions: (i) Is there a geographic component to the distribution of genetic variation of *C. lupus* across Saudi Arabia? (ii) Which subspecies of *C. lupus* is/are present in Saudi Arabia?

Dry skin of road-killed or poached *C. lupus* ($n = 88$ samples), and blood samples from captive individuals ($n = 15$ samples) were collected from across Saudi Arabia (Fig. 1). Provenance is not known for the 15 live, captive animals. Ethics approval for collection of the blood samples from the 15 live individuals came from the Saudi Wildlife Authority through its approval of the Annual Research Plans of the King Khalid Wildlife Research Centre.

DNA extraction was performed using Qiagen extraction reagents and separate spin columns (Epoch Life Science, TX, USA). An ~800 base pair (bp) section of the mitochondrial cytochrome *b* (cytb) gene was amplified using MOLCIT/MVZ primers (Racey et al., 2007). Where this failed, a shorter fragment (~400 bp) was amplified using GVL/H15149 (Irwin et al., 1991; Gaubert et al., 2011). A ~300 bp fragment of the mitochondrial control region (CR) was also amplified using CTRL/H primers (Palomares et al., 2002). PCR was run for 40 cycles at 48 °C and 50 °C annealing temperatures for cytb and CR, respectively. GenBank sequence data for other canids were used for phylogeny construction, including all available *C. lupus* except *C. l. familiaris.* Dog representatives were limited to one from each major clade identified in Pang et al. (2009) and an example from Egypt (accession numbers in Table 1). PCR products, both from cytb and CR regions, were sequenced by Macrogen.

Sequence data were confirmed and aligned in BIOEDIT (version 7.0.5; Hall, 2005). A maximum parsimony haplotype network was calculated using the combined data for both genes in the TCS application (Clement et al., 2000). Diversity measures were calculated using DNASP (version 5.10.01; Librado and Rozas, 2009). Both gene fragments were used to construct a combined phylogeny in MRBAYES 3.1 (Ronquist and Huelsenbeck, 2003). The default models were used for the gene partitions, setting MRBAYES to infer coding bias assuming that only variable characters can be observed for both ("CODING = VARIABLE"). Bayesian analyses were undertaken using four independent runs, each employing a random starting tree and 1×10^6 generations with one cold and three heated chains, sampling trees every 100 generations. The trees were derived from a consensus of the last 9901 trees for the first of the four independent runs (removing the initial 100).

From 40 successfully amplified samples for the cytb fragment, 20 comprised 401 bp and 20 comprised 790 bp. There were six variable sites in the first 401 bp, and 13 across the 790 bp sequences. A control region fragment comprising 282 bp was recovered from 41 individuals, of which there were 17 variable bases. The poor condition of the dry skin samples resulted in amplification failure for one or both genes in 66 samples.

A concatenated dataset of the 37 common individuals for both gene fragments was constructed resulting in 683 bases of comparable sequence. For each of the cytb and control region; haplotype diversity was 0.887 and 0.961, and nucleotide diversity was 0.018 and 0.012 respectively. This was used to produce a haplotype network of the Arabian wolf with three other conspecific representatives for reference (Fig. 2). We found 20 haplotypes for the cytb gene, 13 for the control region, and 24 in the combined fragment. The majority of Arabian wolf haplotypes are closely associated with the other *C. l. lupus* representatives, but at least one divergent haplogroup can be seen (25% of haplotypes with at least 2% sequence divergence from the dog representative). A consensus MRBAYES phylogeny was constructed for the combined dataset (Fig. 3). This phylogeny shows high support (>0.8) for all taxa except for a group comprising all *C. l. lupus* (including Saudi Arabian samples), *C. l. familiaris*, *C. l. campestris*, and one of the *C. l. chanco* samples.