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### Short Communication

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

Timothy C. Bray<sup>a,\*</sup>, Osama Badri Mohammed<sup>a</sup>, Thomas M. Butynski<sup>b,c,1</sup>,  
Torsten Wronski<sup>d</sup>, Mohamed Abdelkader Sandouka<sup>b,c</sup>, Abdulaziz Nasser Alagaili<sup>a,e</sup>

<sup>a</sup> KSU Mammals Research Chair, Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

<sup>b</sup> King Khalid Wildlife Research Centre, Saudi Wildlife Authority, Thumamah, P.O. Box 61681, Riyadh 11575, Saudi Arabia

<sup>c</sup> Conservation Programmes, The Zoological Society of London, Regents Park, London NW1 4RY, United Kingdom

<sup>d</sup> Department of Wildlife & Aquatic Resources Management, College of Agriculture, Animal Sciences and Veterinary Medicine, University of Rwanda, P.O. Box 57, Nyagatare, Rwanda

<sup>e</sup> Saudi Wildlife Authority, P.O. Box 61681, Riyadh 11575, 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.

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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).

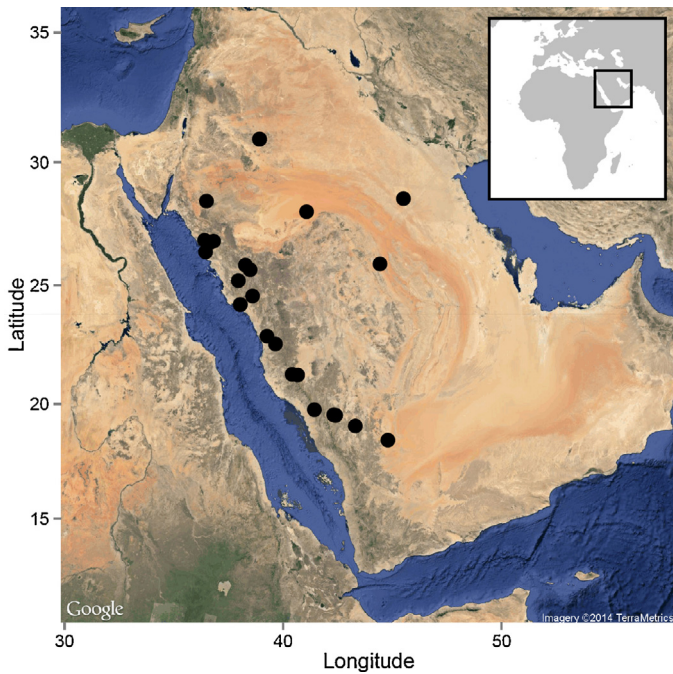
Extensive genetic research has been conducted worldwide across populations of *C. lupus*, but difficulty in comparing studies has arisen due to the use of just one of two mitochondrial genes (rather than both); control region (CR), or cytochrome *b* (cytb), or any of a range of available nuclear microsatellites. 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

\* Corresponding author. Tel.: +966 1146 777 16; fax: +966 1146 785 14.

E-mail addresses: [tim.c.bray@hotmail.com](mailto:tim.c.bray@hotmail.com), [bestialpoke@gmail.com](mailto:bestialpoke@gmail.com) (T.C. Bray).

<sup>1</sup> Current address: Lolldaiga Hills Research Programme, Sustainability Centre Eastern Africa, P.O. Box 149, Nanyuki 10400, Kenya.



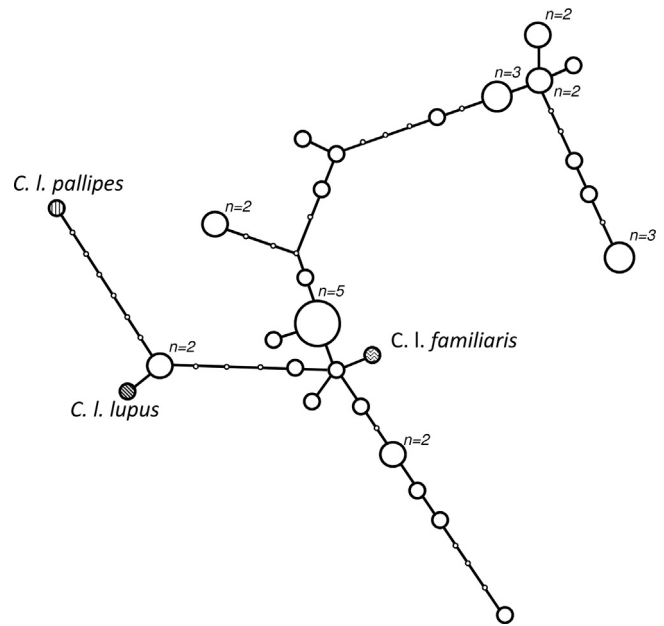
**Fig. 1.** Sampling locations in Saudi Arabia for which *Canis lupus* genetic data were obtained ( $n = 37$ ; image generated in ggmap package in R).

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.



**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).

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  $1 \times 10^6$  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.

**Table 1**  
GENBANK accession numbers and sequence details for the Cytochrome b gene and control region of the canids used in this study.

Species	Identifier	Origin/clade represented	GenBank accession no. Cytb/CR	Reference
<i>C. adustus</i>	<i>C.adustus01</i>	Guinea	JQ088650/JQ088669	Gaubert et al. (2012)
<i>C. adustus</i>	<i>C.adustus02</i>	Guinea	JQ088651/JQ088670	Gaubert et al. (2012)
<i>C. adustus</i>	<i>C.adustus03</i>	Guinea	JQ088652/JQ088671	Gaubert et al. (2012)
<i>C. adustus</i>	<i>C.adustus04</i>	Guinea	JQ088653/JQ088672	Gaubert et al. (2012)
<i>C. adustus</i>	<i>C.adustus05</i>	Guinea	JQ088654/JQ088673	Gaubert et al. (2012)
<i>C. adustus</i>	<i>C.adustus06</i>	Benin	JQ088655/JQ088674	Gaubert et al. (2012)
<i>C. aureus</i>	<i>C.aureus_01</i>	Senegal	JQ088656/JQ088675	Gaubert et al. (2012)
<i>C. aureus</i>	<i>C.aureus_02</i>	Senegal	JQ088657/JQ088676	Gaubert et al. (2012)
<i>C. l. familiaris</i>	<i>Cl.famEGY01</i>	Egypt	JQ088658/JQ088677	Gaubert et al. (2012)
<i>C. lupaster</i>	<i>Cl.lupast01</i>	Algeria	JQ088659/JQ088678	Gaubert et al. (2012)
<i>C. lupaster</i>	<i>Cl.lupast02</i>	Algeria	JQ088660/JQ088679	Gaubert et al. (2012)
<i>C. lupaster</i>	<i>Cl.lupast03</i>	Algeria	JQ088661/JQ088680	Gaubert et al. (2012)
<i>C. lupaster</i>	<i>Cl.lupast04</i>	Algeria	JQ088662/JQ088681	Gaubert et al. (2012)
<i>C. lupaster</i>	<i>Cl.lupast05</i>	Algeria	JQ088663/JQ088682	Gaubert et al. (2012)
<i>C. lupaster</i>	<i>Cl.lupast06</i>	Senegal	JQ088664/JQ088683	Gaubert et al. (2012)
<i>C. lupaster</i>	<i>Cl.lupast07</i>	Mali	JQ088665/JQ088684	Gaubert et al. (2012)
<i>C. lupus</i>	<i>C0012</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>C0027</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>C0028</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>C0034</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>C0035</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>C0044</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>C0124</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>C0125</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W02</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W06</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W07</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W09</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W110</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W121</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W30</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W41</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W42</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W51</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W74</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W75</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W83</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W84</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W88</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>W96</i>	Saudi Arabia	To be confirmed	This study
<i>C. lupus</i>	<i>Cl.NC009686</i>	Sweden	<i>NC009686</i>	Arnason et al. (2007)
<i>C. himalayensis</i>	<i>C.himalay...</i>	India	AY291431/AY289995	Aggarwal et al. (2007)
<i>C. indica</i>	<i>C.indica...</i>	India	AY291432/AY289984	Aggarwal et al. (2007)
<i>C. l. pallipes</i>	<i>C.l.palli01</i>	India	AY333749/AY333746	Sharma et al. (2003)
<i>C. l. pallipes</i>	<i>C.l.palli02</i>	India	AY333749/AY333745	Sharma et al. (2003)
<i>C. l. pallipes</i>	<i>C.l.palli03</i>	India	AY333749/AY333744	Sharma et al. (2003)
<i>C. l. pallipes</i>	<i>C.l.palli04</i>	India	AY333749/AY333743	Sharma et al. (2003)
<i>C. simensis</i>	<i>C.simensis...</i>	Ethiopia	L29416/AY562107	Gotelli et al. (2004)
<i>C. l. lupus</i>	<i>Cl.SPAIN....</i>	Spain	NC.008092	Bjornerfeldt et al. (2006)
<i>C. lupus</i>	<i>Cl.W2.....</i>	Saudi Arabia	EU789787	Pang et al. (2009)
<i>C. lupus</i>	<i>Cl.W5.....</i>	Saudi Arabia	EU789788	Pang et al. (2009)
<i>C. l. lupus</i>	<i>Cl.canada...</i>	Canada	DQ480508	Bjornerfeldt et al. (2006)
<i>C. lupus</i>	<i>Cl.saudi2...</i>	Saudi Arabia	DQ480507	Bjornerfeldt et al. (2006)
<i>C. lupus</i>	<i>Cl.saudi1...</i>	Saudi Arabia	DQ480506	Bjornerfeldt et al. (2006)
<i>C. l. lupus</i>	<i>Cl.SPAIN1...</i>	Spain	DQ480505	Bjornerfeldt et al. (2006)
<i>C. l. lupus</i>	<i>Cl.sweden...</i>	Sweden	DQ480504	Bjornerfeldt et al. (2006)
<i>C. l. lupus</i>	<i>Cl.russia...</i>	Russia	DQ480503	Bjornerfeldt et al. (2006)
<i>C. l. lupus</i>	<i>Cl.japan....</i>	Japan	AB499825	Ishiguro N (unpublished)
<i>C. l. lupus</i>	<i>Cl.china....</i>	China	KC461238	Zhang et al. (2013)
<i>C. l. familiaris</i>	<i>Cl.famil01...</i>	a1	EU789698	Pang et al. (2009)
<i>C. l. familiaris</i>	<i>Cl.famil02...</i>	a2	EU789678	Pang et al. (2009)
<i>C. l. familiaris</i>	<i>Cl.famil03...</i>	a3	EU789672	Pang et al. (2009)
<i>C. l. familiaris</i>	<i>Cl.famil04...</i>	a4	EU789759	Pang et al. (2009)
<i>C. l. familiaris</i>	<i>Cl.famil05...</i>	a5	EU789669	Pang et al. (2009)
<i>C. l. familiaris</i>	<i>Cl.famil06...</i>	a6	EU789668	Pang et al. (2009)
<i>C. l. familiaris</i>	<i>Cl.famil07...</i>	b1	EU789785	Pang et al. (2009)
<i>C. l. familiaris</i>	<i>Cl.famil08...</i>	b2	EU789651	Pang et al. (2009)
<i>C. l. familiaris</i>	<i>Cl.famil09...</i>	c1	EU789660	Pang et al. (2009)
<i>C. l. familiaris</i>	<i>Cl.famil10...</i>	c2	EU789772	Pang et al. (2009)
<i>C. l. campestris</i>	<i>Cl.campest...</i>	Mongolia	KC896375	Zhang et al. (2013)
<i>C. l. chanco</i>	<i>Cl.chanco01</i>	China	NC.010340	Meng et al. (unpublished)
<i>C. l. chanco</i>	<i>Cl.chanco02</i>	China	EU442884	Meng et al. (unpublished)
<i>C. l. chanco</i>	<i>Cl.chanco03</i>	China	GC374438	Chen L and Zhang HH (unpublished)
<i>C. l. laniger</i>	<i>Cl.laniger...</i>	Tibet	NQ.011218	Pang et al. (2009)
<i>C. latrans</i>	<i>C.latrans01</i>	USA	EU789789	Bjornerfeldt et al. (2006)

Table 1 (Continued)

Species	Identifier	Origin/clade represented	GenBank accession no. Cytb/CR	Reference
<i>C. latrans</i>	<i>C.latrans02</i>	USA	DQ480511	Bjornerfeldt et al. (2006)
<i>C. latrans</i>	<i>C.latrans03</i>	USA	DQ480510	Bjornerfeldt et al. (2006)
<i>C. latrans</i>	<i>C.latrans04</i>	USA	DQ480509	Meng et al. (2009)
<i>Cuon alpinus</i>	<i>Cuon.alpinus</i>	China	NC_013445	Wayne et al. (1997) and Marsden et al. (2012)
<i>Lycaon pictus</i>	<i>Lycaon.pictus</i>	Africa	AF028147/JJQ282690	

*Is there a geographic component to the distribution of genetic variation of C. lupus across Saudi Arabia?*

Within Saudi Arabia, the samples for *C. lupus* show no clear genetic discontinuity. This is not unexpected for such a highly vagile animal; data for *C. lupus* in a comparable habitat suggest a home range of up to 60 km<sup>2</sup> (Afik and Alkon, 1983), with dispersal up to 200 km (Hefner and Geffen, 1999).

*Which subspecies of C. lupus is/are present in Saudi Arabia?*

Whilst the Saudi Arabian contribution to the phylogeny of *C. lupus* is spread throughout, there are two main clusters; one containing no other type and one containing the Egyptian dog sample (Egyptian dog marked with an asterisk; Fig. 3). Although with poor support, at the very least, these two clusters might represent subpopulations for future studies of *C. lupus* in Saudi Arabia. The samples used in this study are mainly from animals killed on the road or shot. As such, it is possible (although perhaps unlikely) that the dataset has a bias towards *C. lupus* that were associated with humans and, thereby, with *C. l. familiaris*.

Despite increasing human expansion across much of Saudi Arabia, there are still large areas where wildlife exists apart from humans. Whilst some of the genetic variation seen in this study can be attributed to interbreeding with *C. l. familiaris*, there appears to be a considerable element that is distinct from this influence. Our

results indicate that the *C. lupus* sampled in this study are not *C. l. pallipes*, but fall within the spectrum of diversity of the Eurasian *C. l. lupus* group.

Recent taxonomies have subsumed *C. l. arabs* within *C. l. lupus* (Rueness et al., 2011; Gaubert et al., 2012). The unique genetic variation observed in this study indicates, however, that the *C. lupus* of Saudi Arabia should be retained within *C. l. arabs* (Pocock, 1934; Harrison and Bates, 1991) until a more in-depth assessment is made. We cannot categorically state that *C. l. pallipes* is absent from Saudi Arabia, in fact none of the sampled wolves align with this subspecies.

One result of this phylogenetic analysis is the strong distinction between *C. lupus* of Saudi Arabia and *C. l. pallipes*, and the close association with *C. l. lupus* (Fig. 3). While there could be an argument for extensive introgression from *C. l. familiaris* resulting in the artificial proximity to the Eurasian *C. l. lupus* group rather than to *C. l. pallipes*, the high genetic variation found in *C. lupus* of Saudi Arabia suggests either extensive long-term introgression, or recent introgression combined with recent co-ancestry. Several lineages of the Saudi Arabian samples are distinct from any of the *C. l. familiaris* included here. Although not well supported, this suggests genetic variation separate from known *C. l. familiaris* sources and extensive isolation in some lineages.

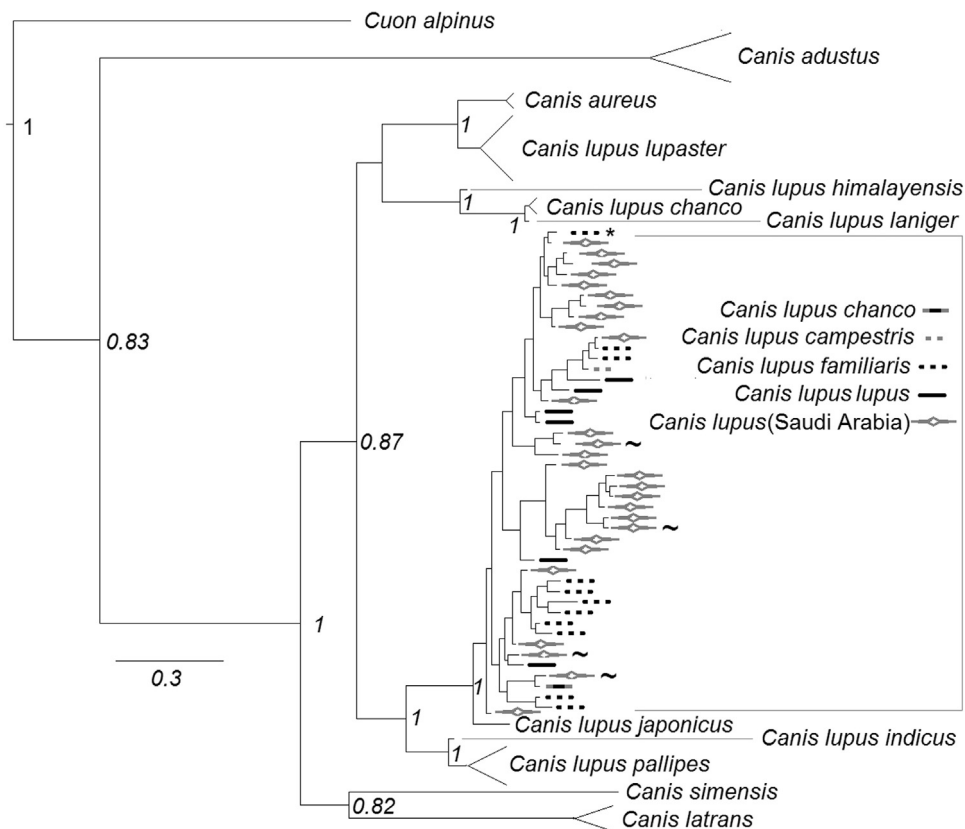


Fig. 3. *Canis lupus* consensus MRBAYES phylogeny for Saudi Arabia using the combined cytb and CR dataset. The four additional Arabian wolf samples of unknown origin are marked with a tilde, the Egyptian dog sample is marked with an asterisk. Only bootstrap support values for nodes above 0.8 shown.

In contrast with the most recent *C. lupus* phylogeny for this region (Gaubert et al., 2012), this study found that GenBank representatives of *Canis lupus* do not give a clear cut phylogeny. At least one representative of the Tibetan wolf (*C. l. chanco*) falls within the Eurasian *C. l. lupus* group, and the MRBAYES phylogeny suggests that the sample from Japan is distinct from *C. l. lupus*. More balanced sampling of each *C. l. lupus* subregion, as well as a more extensive representation of nuclear and mitochondrial gene regions, will provide greater resolution in the *C. l. lupus* puzzle.

The availability of genetic data for comparison is limited in *C. lupus* in Saudi Arabia. This is due, in part, to the unavailability of much of the important information (e.g., Savolainen et al., 2002). The majority of data for *C. lupus* in Saudi Arabia that has been used in past studies are notably unreferenced and no longer available for further analysis (see Vilà et al., 1999; Sharma et al., 2004). Recent studies (e.g., Cohen et al., 2013) mention data for *C. l. arabs*, but these are based on putative species distributions and historical ranges.

We have gone some way towards describing the genetic variation of *C. lupus* in Saudi Arabia using the most commonly employed gene regions. Nonetheless, a consensus application of microsatellite, SNP, and/or another comparable and highly variable genetic marker set, is necessary for this species. In particular, for considering hybridization, both nuclear and mitochondrial markers are essential. Whilst it seems likely that there is an overlap between the gene pools of *C. lupus arabs* and *C. lupus familiaris* in Saudi Arabia, it remains to be determined whether this is the source of the relatively high genetic diversity seen in this region. A rapidly evolving nuclear marker dataset would also give an alternative estimate on the suggested population size of ~500 individuals (Mech and Boitani, 2004), as well as much-needed insight into changes in population size over time, of one of the few remaining large carnivores in Saudi Arabia.

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