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Ancient wolf lineages in India

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All previously obtained wolf (*Canis lupus*) and dog (*Canis familiaris*) mitochondrial (mt) DNA sequences fall within an intertwined and shallow clade (the 'wolf-dog' clade). We sequenced mtDNA of recent and historical samples from 45 wolves from throughout lowland peninsular India and 23 wolves from the Himalayas and Tibetan Plateau and compared these sequences with all available wolf and dog sequences. All 45 lowland Indian wolves have one of four closely related haplotypes that form a well-supported, divergent sister lineage to the wolf-dog clade. This unique lineage may have been independent for more than 400 000 years. Although seven Himalayan wolves from western and central Kashmir fall within the widespread wolf-dog clade, one from Ladakh in eastern Kashmir, nine from Himachal Pradesh, four from Nepal and two from Tibet form a very different basal clade. This lineage contains five related haplotypes that probably diverged from other canids more than 800 000 years ago, but we find no evidence of current barriers to admixture. Thus, the Indian subcontinent has three divergent, ancient and apparently parapatric mtDNA lineages within the morphologically delineated wolf. No haplotypes of either novel lineage are found within a sample of 37 Indian (or other) dogs. Thus, we find no evidence that these two taxa played a part in the domestication of canids.

Keywords: *Canis lupus*; India; wolf; mitochondrial DNA; evolution

1. INTRODUCTION

Recent mitochondrial (mt) DNA analyses of wolves and dogs revealed that wolves, throughout their range, are genetically very similar and that they and dogs share a recent common ancestor (Tsuda *et al.* 1997; Vila *et al.* 1997; Leonard *et al.* 2002; Savolainen *et al.* 2002). Although over 32 wolf subspecies have been described (Mech 1970), most subspecies differences are not upheld by genetic analysis, and phylogeographic structure is weak (Vila *et al.* 1999). Wolves in peninsular India are part of the subspecies *Canis lupus pallipes*, which ranges from the Indian subcontinent to the Arabian Peninsula (Nowak 1995; figure 1). Wolves throughout this region are smaller than other Eurasian wolves and adapted to desert conditions (Nowak 1995; Jhala 2000). All sequences from

pallipes wolves from the Middle East ($n = 40$) fall within the wolf-dog clade (Tsuda *et al.* 1997; Vila *et al.* 1997, 1999; Savolainen *et al.* 2002). We use mtDNA sequences to assess the phylogeography of wolves from the Indian subcontinent and their relationships to other wolf subspecies and canids.

2. MATERIAL AND METHODS

(a) Samples and sequences

We obtained mtDNA control region (CR) sequences for 45 *C. l. pallipes* from throughout most of their current range in the subcontinent and 23 *C. l. chanco* from the western and central Himalayas (figure 1b; electronic Appendix B, available on The Royal Society's Publications Web site). Most samples were obtained from an ongoing study at the Wildlife Institute of India, but 20 sequences were obtained from museum specimens. We compared these sequences with all of the wolf and dog sequences currently available in GenBank (254 haplotypes from 453 different sequences from more than 700 individuals), which include 40 individual *C. l. pallipes* from the Middle East (Afghanistan to Turkey) and 22 *C. l. chanco* from Mongolia and China (figure 2b). Cytochrome *b* (cyt *b*) sequences were obtained from 17 *C. l. pallipes* and 14 *C. l. chanco*. (See electronic Appendix A for detailed laboratory methods.)

(b) Sequence analysis

Maximum-likelihood, maximum-parsimony and neighbour-joining trees were constructed with PAUP* v. 4.0b10 (Swofford 2002). To select the model of DNA substitution that best fitted the data that we used, a hierarchical likelihood-ratio test approach implemented in the program MODELTEST 3.06 (Posada & Crandall 1998). A (HKY+I+G) model (where HKY is the Hasegawa-Kishino-Yano 85 distance correction, I is invariant sites and G is gamma parameter) was selected for CR with $ts:tv = 17.32$; $I = 0.417$ and $G = 0.336$. We used molecular evolutionary genetics analysis (MEGA) (Kumar *et al.* 2000) to calculate divergences and rates. Because MEGA does not contain an HKY model, we used the similar Tamura-Nei model, which produced slightly higher distances. Confidence in estimated relationships was determined using 1000 bootstrap pseudo-replicates (Swofford 2002). The maned wolf (*Chrysocyon brachyurus*), simien jackal (*Canis simensis*), coyote (*Canis latrans*) and golden jackal (*Canis aureus*) were used as potential sister groups and outgroups based on earlier phylogenetic analysis of canids using morphological and mtDNA characters (Wayne *et al.* 1997).

3. RESULTS

We found only four haplotypes among the 45 *C. l. pallipes* CR sequences in India and Pakistan, each differing by 1–4 substitutions, and only one cyt *b* haplotype. Surprisingly, phylogenetic analyses of the CR (figure 2a) and cyt *b* sequences reveal that these are not part of the wolf-dog clade, but rather form a well-supported sister clade to it. All 40 *C. l. pallipes* sequences from areas to the west of India and Pakistan (i.e. Iran, Israel, Afghanistan, Saudi Arabia and Turkey) fall within the wolf-dog clade. Average Tamura-Nei+G distances between the Indian and wolf-dog clade, corrected for intraclade variation, are $6.1 \pm 1.4\%$ and $1.3 \pm 0.5\%$ for CR and cyt *b*, respectively. Evidence indicates that these represent mtDNA differences, and not nuclear copies of mtDNA (NUMT) (see electronic Appendix A).

Out of the Himalayan *C. l. chanco*, seven sequences from Kashmir fell within the wolf-dog clade (figure 2b; electronic Appendix B). However, our easternmost Kashmir sample, nine from Spiti Valley in Himachal Pradesh, four from Nepal and two from Tibet had CR and cyt *b* haplotypes that were highly divergent from all other wolf-dog sequences ($12.0 \pm 4.0\%$ and $3.2 \pm 0.9\%$, respectively). These sequences form a basal clade within *Canis lupus* (figure 2a).

We used fossil record estimates of the divergence time of the coyote and wolf lineages to calibrate sequence divergence rates for each gene using MEGA (Kumar *et al.*

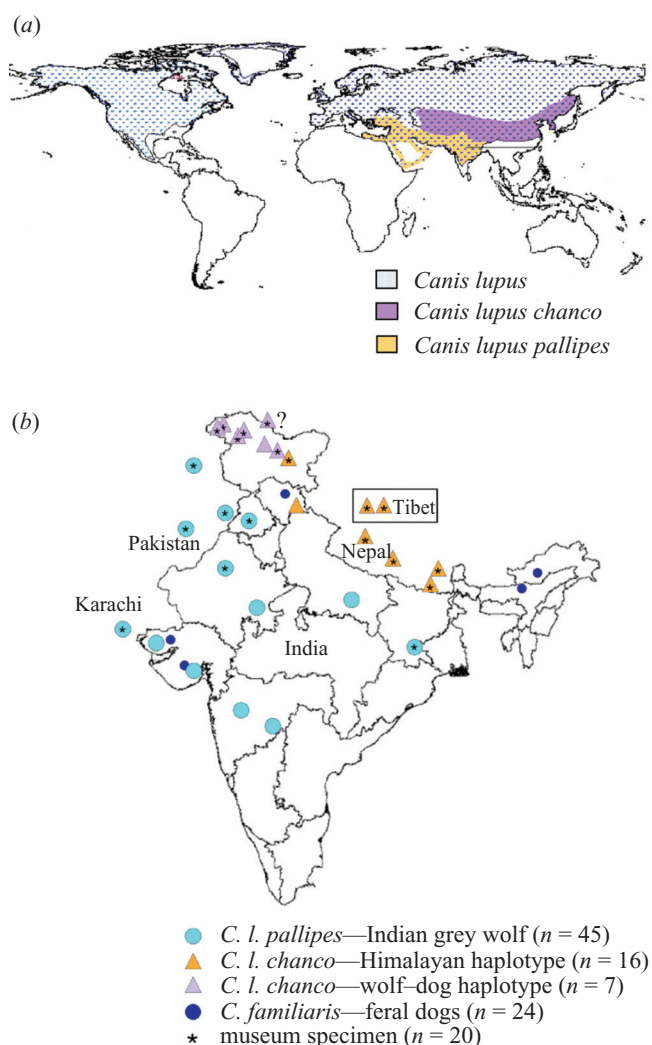


Figure 1. (a) Map of wolf distribution in the Northern Hemisphere showing historical distributions of *Canis lupus*, and the subspecies *C. l. pallipes* and *C. l. chanco*. (b) Map of the Indian subcontinent with study sampling localities indicated for wolves and dogs. No further locality data beyond country were available for two Tibet (in box) and one Nepal sample (below box). The question mark refers to a USNM sample that was of uncertain origin; field notes suggest it was collected in Ladakh, Kashmir.

2000). Fossil evidence suggests that anatomically modern wolves and coyotes have existed for about 1 million years (Myr), while putative ancestral lineages of each may have existed for twice that long (Nowak 1979; Savolainen *et al.* 2002). Thus, we used 1 and 2 Myr to bracket the time depth of the split. Cyt *b* branch lengths did not fail a likelihood-ratio test of equal rates using PAUP* ($G = 22.8$, 14 d.f., $p > 0.05$), however, CR did fail ($G = 81.0$, 52 d.f., $p < 0.01$). The cyt *b* rate calculated in MEGA (Kumar *et al.* 2000) for a 1 Myr split was $2.1\% \text{ Myr}^{-1}$ and the CR rate was $9.2\% \text{ Myr}^{-1}$. For Indian *C. l. pallipes* versus wolf–dog clade, MEGA yielded corrected divergence times of 0.4 (1 Myr split) and 0.8 Myr (2 Myr split) for both cyt *b* and CR (more than double the entire coalescence time for the wolf–dog clade at 0.14 or 0.28 Myr). MEGA yielded corrected divergence times between the very different Himalayan clade and the wolf–

dog clade of *ca.* 0.8 and 1.5 Myr, respectively, for both cyt *b* and CR.

4. DISCUSSION

Our results suggest that there has been no mtDNA gene flow between the wolf–dog clade and the Indian *pallipes* clade in peninsular India. Furthermore, the discovery of the geographically separate Himalayan clade, occurring from eastern Nepal and Tibet to eastern Kashmir, suggests that an ancient lineage is still extant in these higher elevation regions. These three clades appear to have diverged in the mid-Pleistocene (0.5–1.5 Myr ago) and to be distributed parapatrically. The potential area of contact between them is in Kashmir (figure 1).

Worldwide genetic structure indicates that wolves have had high gene flow within and between the New and Old Worlds (Vila *et al.* 1999), attributed to their great dispersal capabilities (Gese & Mech 1991). Thus, the apparent lack of mtDNA introgression among wolves of these three clades is surprising. The Indian *C. l. pallipes* distribution corresponds largely to the peninsular Indian division of the Indian subregion, in which there are endemic genera and species of mammals, including antelopes and rodents (Corbet & Hill 1992). It inhabits arid and semiarid grasslands and scrub forest (Jhala & Giles 1991) and is geographically separated from *C. l. chanco* by the Terai swamp grasslands and Himalayan foothills, a region apparently devoid of wolves (Hodgson 1847). We speculate that behavioural (Harrington & Paquet 1982), ecological (Jhala & Giles 1991) or morphological (Blanford 1888; Nowak 1995) differences between Indian *C. l. pallipes* and members of the wolf–dog and Himalayan lineages could prevent interbreeding.

Given the high dispersal capabilities of wolves, and the proximity of their ranges, there would appear to be no physical barriers to gene flow between *C. l. chanco* of the Himalayan and wolf–dog clades. Past geological events in the region may have influenced the current phylogeographic patterns that we observe, and studies of mtDNA from sub-fossil wolf bones might allow us to reject the possibility that these patterns are the result of chance dispersal events causing changes of clade dominance over time (e.g. Barnes *et al.* 2002). Interestingly, the estimated time of the split of the Himalayan wolf from the other wolf lineages (0.8–1.5 Myr ago; figure 2a) correlates with the period of rapid uplift of the Tibetan Plateau and associated habitat modification (0.9–1.1 Myr ago; Sun & Liu 2000).

We propose two historical scenarios to explain these clade distributions: (i) several ancient wolf lineages were preserved in South Asia while all but the wolf–dog lineage went extinct elsewhere in the world or; and more parsimoniously, (ii) wolves evolved in South Asia and only the wolf–dog lineage spread out of this region. Conceivably, during Pleistocene glaciations these ancient wolf lineages were isolated in refuges in peninsular India, and their relatively low genetic diversity could have resulted from the expansion of small refugial populations.

The systematics of wolves from the Indian subcontinent has been poorly studied and remains controversial. Early taxonomists noted that both the Indian *C. l. pallipes* and the Himalayan *C. l. chanco* were distinct relatives to other wolves. Hodgson (1847) described the Himalayan wolf as

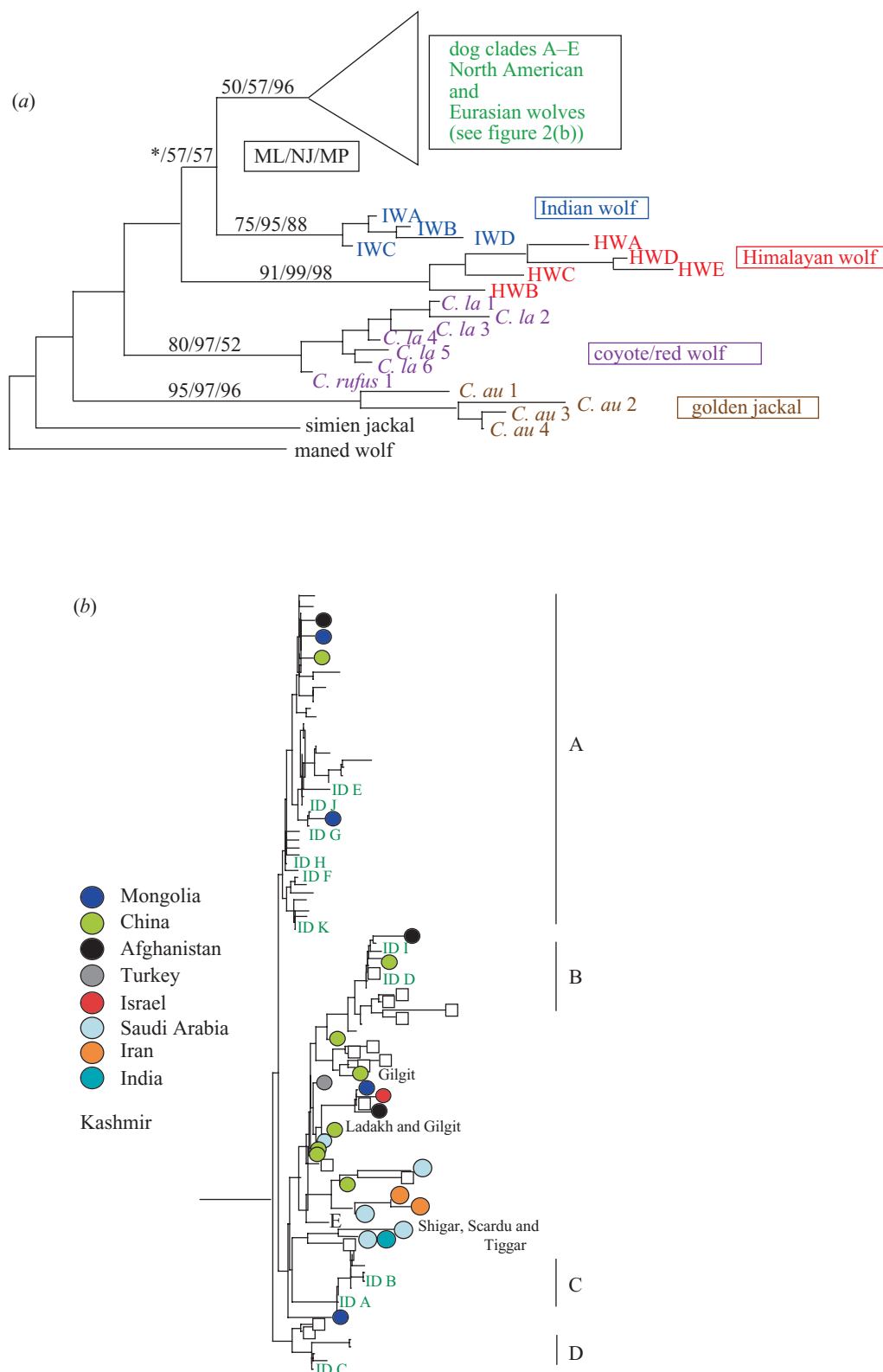


Figure 2. (a) Neighbour-joining tree constructed from HKY+I+G corrected distances among control region sequences of canids. This includes a broad sampling of previously published wolf–dog clade sequences of *C. lupus* and *C. familiaris* shown as a triangle (see § 2a and part (b) of the figure) and sequences from peninsular Indian *C. l. pallipes* ($n = 45$), Himalayan and Kashmir *C. l. chanco* ($n = 23$), golden jackal, simien jackal, coyote and red wolf. The outgroup is maned wolf. Bootstrap support is indicated at nodes if found in more than 50% of 1000 replicates from maximum-likelihood, neighbour-joining and maximum-parsimony analyses. The tree was constructed in PAUP* v. 4.0b10 (Swofford 2002). An identical topology was obtained from the smaller cyt *b* dataset (results not shown). (b) Expanded topology of the wolf–dog clade. Five clades (A–E) as in Savoleinen *et al.* (2002) are indicated. Previously published dog haplotypes (unlabelled) and wolf haplotypes (open squares) as well as 11 haplotypes of Indian dogs from this study (ID A–K) are indicated. Previously published sequences of *C. l. chanco* and *C. l. pallipes* (coloured circles) are shown for comparison with our seven sequences from Kashmir.

a distinct species, *C. laniger*, noting its well-developed frontal sinuses, unusual elongation of the muzzle, distinct coloration and the woolliness of its underfur. CR sequence obtained from a specimen that he collected (BM58.6.24.61) fell within the Himalayan clade. Blanford (1888) later combined *C. laniger* with *C. lupus*, and elevated the Indian wolf to *C. pallipes*. Much later, Pocock (1941) described both taxa as subspecies of *C. lupus*, making *C. laniger* and *C. pallipes* parts of the more widely distributed *C. l. chanco* and *C. l. pallipes*, respectively (figure 1a). Pocock was influenced by intermediate pelaged individuals at areas of contact in northern Punjab and Kashmir. Our data support Hodgson's *C. laniger* and Blanford's *C. pallipes*. However, finer-scale studies in areas of potential contact (Kashmir and Punjab) and analyses with nuclear markers are needed to rule out the possibility that these patterns are the result of strong female philopatry in the presence of moderate rates of male gene flow (Paetkau *et al.* 1998). Nonetheless, extensive radio-tracking studies (Y. Jhala, unpublished data) did not reveal differential dispersal by sex in Indian wolves.

We also sequenced 24 feral dogs from areas in India where contact between them and wolves is common (Jhala & Giles 1991). We found 11 haplotypes that fall within the wolf-dog clade (figure 2b), as did six haplotypes from 13 Indian dogs described in a recent mtDNA survey of Asian dogs (Savolainen *et al.* 2002). Our results suggest that 'Bhutia', 'Twang', Tibetan Mastiff and local 'pariah' dog breeds were brought into the Himalayas and peninsular India by humans, and were not domesticated independently from Indian *C. l. pallipes* or Himalayan *C. l. chanco*. It seems likely that South Asia is not the region of origin for the domestic dog (Savolainen *et al.* 2002).

Indian *C. l. pallipes* are endangered; their range covers most of peninsular India, and their population is estimated at 2000–3000 (Jhala 2000). Conservation efforts for this taxon merit greater priority given its unique and ancient genetic lineage. The possible current distribution of the mtDNA lineage of Himalayan *C. l. chanco* extends from eastern Kashmir into eastern Nepal and Tibet. Almost nothing is known about the ecology, behaviour and status of these wolves. Persecution of wolves by pastoralists is common in this region. Our study suggests that this genetically unique wolf also deserves high priority for conservation and further research.

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