

These are electronic appendices to the Biology Letter by Kumar *et al.* 2004 Ancient wolf lineages in India. *Proc. R. Soc. Lond. B (Suppl.)* **271**, S1–S4. (DOI 10.1098/rsbl.2003.0071.)

Electronic appendices are refereed with the text. However, no attempt has been made to impose a uniform editorial style on the electronic appendices.

Electronic Appendix A

DNA Methods:

Modern DNA was extracted from hair, blood, and tissue samples using a QIAGEN DNeasy™ DNA extraction kit following manufacturer's protocol (Qiagen, Valencia, CA). Ancient DNA was extracted from samples taken from museum specimens that included 22 bone samples and one skin sample. These were obtained from the Natural History Museum, South Kensington, UK (BM; n=14), the National Museum of Natural History, Washington, DC, USA (NMNH; n= 8), and the Field Museum of Natural History, Chicago, IL, USA (FMNH; n =1). Specimens had been collected in current-day Nepal, Tibet, India and Pakistan. In some cases, exact locality data were not available; these are noted in online appendix B. DNA extractions were performed as in Fleischer *et al.* (2000) and all but two specimens yielded sequence. BM samples were analyzed in an ancient DNA lab at the University of Durham in the UK, while FMNH and USNM samples were analyzed at the Smithsonian Genetics Program ancient DNA laboratory in the U.S.

We amplified two regions of mitochondrial DNA from modern samples: an approximately 440 base pair (bp) fragment of the control region I (CR) using universal primers ThrL 15926 and DL-H 16340 as in Vilà *et al.* (1999); and a 412 bp fragment of the cytochrome *b* gene using a canid specific light primer (Canid L1, Paxinos *et al.* 1997) and a universal heavy primer (H15915, Kocher *et al.* 1989).

Each PCR reaction was carried out in a 25µl volume including 0.5 units of AmpliTaq Gold (Perkin-Elmer), 2.5 mM MgCl₂, 1X reaction buffer (Perkin-Elmer) 200µM each dNTP, and 1µM each primer. Mineral oil was added. Reactions were run for 5 minutes at 94°C and subsequently for 35 cycles of 1 minute denaturing at 92°C, 1.5 minute annealing at 48-50°C and 1.5 minutes extension at 72°C and a final extension cycle at 72°C for 7 minutes in a 480 PE thermocycler (Perkin-Elmer). All DNA extractions and PCR amplification from modern Indian wolves were performed at the Conservation Genetics Laboratory at the WII. Gel purified PCR products were directly sequenced with the Big Dye terminator chemistry and resolved on an ABI 377 automated sequencer (Perkin-Elmer) at the Smithsonian Institution Genetics Program.

For "ancient" samples we designed internal primer sequences to amplify shorter fragments of the CR: IWDL220R (5'-GTTTCTCGAGGCATGGTGAT-3'); IWDL220F (5'-TGAATCACCCCTACTGTGCTAT-3'); IWDL150F (5'-GGTTTGCCCCATGCATATAA-3') and IWDL206R (5'-AAGCCCTTATTGGACTAGGTGA-3'). PCR amplifications used AmpliTaq gold (Perkin-Elmer) and reaction conditions as in Fleischer et al. (2000). PCR products were directly sequenced as described above. Sequence data were aligned using the program SEQUENCHER 4.12 (Gene Codes Corp.).

Nuclear Transpositions:

We do not feel that the three different clades of wolves found in our study were an artifact of nuclear transpositions of mitochondrial DNA for the following reasons:

1. We cloned PCR products from three individuals used in this study – one

Himalayan wolf (D192 from Himachal Pradesh), one Indian wolf (D107 from Bhal) and one Indian dog (D9P, Bhutia dog from Himachal Pradesh). We sequenced from five clones per individual. In all cases, the clone sequences matched those expected based on the earlier direct sequence, with the exception of a small number of single base changes (0.43 mutations per sequence on average) expected from such cloning experiments. In no case did a sequence arise that matched one from a different clade.

2. It is not likely that two separate amplifications of different gene regions (Cytb and CR) would both result in nuclear copies. Both pieces show the same pattern and relative levels of divergence.
3. It is unlikely that all of the smaller, often overlapping pieces of sequence amplified from museum specimens with different primers would be nuclear copies. We did not detect sequences from one clade that matched those of another among these pieces, something that would suggest nuclear copies.
4. We did not see any consistent double sequence.
5. In the Cytb sequences, we did not find stop codons, insertions or deletions, or other unusual patterns often suggestive of nuclear copies.

Wolf and Dog Hybridization:

Although there is some indication that Indian *C. l. pallipes* can breed with dogs in captivity, we found no evidence of natural hybridization (i.e., wolf haplotypes in Indian dogs, or vice-versa) unlike that occasionally observed in Europe (Randi & Lucchini 2002). However, our sample sizes are relatively small for this type of analysis, and a

frequency of 6.4% of dog haplotypes would have to be present in the wolf population in order to be detected in our sample of 45 wolves (95% CI from a binomial sampling distribution). In addition, all of our wolf sequences that fall within the dog-wolf clade have haplotypes identical or very closely related to previously published wolf sequences from China and Saudi Arabia, are nested within a clade only containing wolf haplotypes (Fig 1b), and do not fall in any of the major dog clades (A, B, C, D and E) (Tsuda et al, Vila et al Savolainen et al). All of our Indian dog sequences, however, fall in several of the four major dog clades reported in Savolainen et al (2002), but not in the wolf clade.

Literature Cited:

- Fleischer, R. C., Olson, S., James, H. F. & Cooper, A. C. 2000. The identity of the extinct Hawaiian eagle (*Haliaeetus*) as determined by mitochondrial DNA sequence. *Auk* 117, 1051-1056.
- Kocher, T.D., Thomas W.K., Meyer A. *et al.* 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers *Proc. Natl. Acad. Sci. USA* 86, 6196-6200.
- Paxinos E., McIntosh C., Ralls K. & Fleischer R. 1997. A noninvasive method for distinguishing among canid species: amplification and enzyme restriction of DNA from dung. *Mol. Ecol.* 6, 483-486.
- Randi, E. & Lucchini, V. 2002. Detecting rare introgression of domestic dog genes into wild wolf (*Canis lupus*) populations by Bayesian admixture analyses of microsatellite variation *Cons. Gen.* 3, 29 (2002).

Electronic Appendix B

Specimen				Genbank			
ID	Species	Sex	Sample	Locality	Haplotype acc.#	Date	Collector
Samples of wolves obtained from the field							
D1	<i>Canis lupus pallipes</i>	m	ear pinna	Velavadar National Park, Gujarat	IW-A AY333743	18-Jan-95	Y. V. Jhala
D103	<i>Canis lupus pallipes</i>	f	blood	Bhal, Gujarat outskirts of Velavadar National park	IW-A AY333743	25-Nov-96	Y. V. Jhala
D105	<i>Canis lupus pallipes</i>	unk	skin+hair	Nanaj-Maharashtra, Great Indian Bustard sanctuary	IW-C AY333745	17-Jun-99	Satish K. Sharma
D110	<i>Canis lupus pallipes</i>	m	skin+hair	Eastern Uttar Pradesh, Jaunpur district.	IW-B AY333744	15-Oct-96	Y. V. Jhala
D111	<i>Canis lupus pallipes</i>	f	tissue	Kachchh district of Gujarat, in Abdasa Taluka	IW-B AY333744	02-Jan-97	Y. V. Jhala
D113	<i>Canis lupus pallipes</i>	m	tissue	Eastern Uttar Pradesh, Jaunpur district.	IW-B AY333744	11-Nov-96	D. K. Sharma
D114	<i>Canis lupus pallipes</i>	m	tissue	Eastern Uttar Pradesh, Pratapgadh District	IW-B AY333744	09-Jan-96	Y. V. Jhala
D115	<i>Canis lupus pallipes</i>	f	blood	Bhal, Gujarat outskirts of Velavadar National park	IW-A AY333743	23-Feb-96	Y. V. Jhala
D118	<i>Canis lupus pallipes</i>	m	tissue	Bhal, Gujarat outskirts of Velavadar National park	IW-B AY333744	21-Feb-96	Y. V. Jhala
D119	<i>Canis lupus pallipes</i>	m	tissue	Eastern Uttar Pradesh, Pratapgadh District	IW-A AY333743	01-Nov-96	D. K. Sharma
D121	<i>Canis lupus pallipes</i>	f	blood	Kachchh district of Gujarat, Daun Grasslands	IW-A AY333743	18-Feb-97	Y. V. Jhala
D151	<i>Canis lupus pallipes</i>	m	Blood	Kachchh district of Gujarat, in Abdasa Taluka	IW-A AY333743	20-Jun-99	Y. V. Jhala
D163	<i>Canis lupus pallipes</i>	f	Blood	Bhal, Gujarat outskirts of Velavadar National park	IW-A AY333743	27-Dec-00	Y. V. Jhala
D166	<i>Canis lupus pallipes</i>	f	ear pinna	Kachchh district of Gujarat, Daun Grasslands	IW-B AY333744	04-Dec-00	Y. V. Jhala
D19	<i>Canis lupus pallipes</i>	f	muscle	Kachchh district of Gujarat, Daun Grasslands	IW-A AY333743	02-Jul-00	Y. V. Jhala
D2	<i>Canis lupus pallipes</i>	m	blood	Hindustan Aeronautics Ltd Air Base, Nasik, Maharashtra	IW-A AY333743	17-Apr-99	Y. V. Jhala
D25	<i>Canis lupus pallipes</i>	m	blood	Hindustan Aeronautics Ltd Air Base, Nasik, Maharashtra	IW-A AY333743	13-Jun-00	Y. V. Jhala
D32	<i>Canis lupus pallipes</i>	unk	blood	Bhal, Gujarat outskirts of Velavadar National park	IW-A AY333743	19-Jun-99	Y. V. Jhala
D4	<i>Canis lupus pallipes</i>	f	blood	Hindustan Aeronautics Ltd Air Base, Nasik, Maharashtra	IW-A AY333743	17-Apr-99	Y. V. Jhala
D48	<i>Canis lupus chanco</i>	m	tissue	Laddakh, Jammu and Kashmir, India	W15 AF008142	20-Jun-99	R. S. Chundawat
D5	<i>Canis lupus pallipes</i>	f	blood	Kachchh district of Gujarat, Daun Grasslands	IW-A AY333743	01-Aug-99	Y. V. Jhala
D50	<i>Canis lupus pallipes</i>	m	blood	Bhal, Gujarat outskirts of Velavadar National park	IW-A AY333743	02-Jul-95	Y. V. Jhala
D54	<i>Canis lupus pallipes</i>	m	blood	Kachchh district of Gujarat, Abdasa Taluka, in Pat River catchment.	IW-B AY333744	02-Aug-98	Y. V. Jhala
D57	<i>Canis lupus pallipes</i>	m	blood	Velavadar National Park, Gujarat	IW-A AY333743	29-Dec-97	Y. V. Jhala
D59	<i>Canis lupus pallipes</i>	m	Blood	Velavadar National Park, Gujarat	IW-A AY333743	31-Jan-95	Y. V. Jhala
D63	<i>Canis lupus pallipes</i>	f	blood	Kachchh district of Gujarat, Daun Grasslands	IW-B AY333744	01-Aug-99	Y. V. Jhala
D69	<i>Canis lupus pallipes</i>	m	tissue	Eastern Uttar Pradesh, Pratapgadh District	IW-B AY333744	15-Oct-96	Y. V. Jhala
D73	<i>Canis lupus pallipes</i>	f	tissue	Kachchh district of Gujarat, in Abdasa Taluka	IW-A AY333743	11-Apr-97	Y. V. Jhala
D75	<i>Canis lupus pallipes</i>	f	tissue	Kachchh district of Gujarat, in Abdasa Taluka	IW-A AY333743	04-Aug-97	Y. V. Jhala

D76	<i>Canis lupus pallipes</i>	m	gum-tissue	Kachchh district of Gujarat, in Abdasa Taluka	IW-B	AY333744	01-Aug-99	Y. V. Jhala
D79	<i>Canis lupus pallipes</i>	m	blood	Hindustan Aeronautics Ltd Air Base, Nasik, Maharashtra	IW-A	AY333743	17-Apr-99	Y. V. Jhala
D81	<i>Canis lupus pallipes</i>	m	tissue	Kachchh district of Gujarat, in Abdasa Taluka	IW-A	AY333743	28-Jan-99	Y. V. Jhala
D82	<i>Canis lupus pallipes</i>	f	Ear pinna	Kachchh district of Gujarat, Daun Grasslands	IW-A	AY333743	01-Aug-99	Y. V. Jhala
D87	<i>Canis lupus pallipes</i>	f	blood	Kachchh district of Gujarat, in Abdasa Taluka	IW-A	AY333743	05-Oct-98	Y. V. Jhala
D89	<i>Canis lupus pallipes</i>	f	Ear pinna	Bhal, Gujarat outskirts of Velavadar National park	IW-A	AY333743	23-Feb-96	Y. V. Jhala
D94	<i>Canis lupus pallipes</i>	m	tissue	Kachchh district of Gujarat, in Abdasa Taluka	IW-A	AY333743	02-Sep-97	Y. V. Jhala
D97	<i>Canis lupus pallipes</i>	m	ear pinna	Bhal, Gujarat outskirts of Velavadar National park	IW-A	AY333743	02-Jul-95	Y. V. Jhala
D98	<i>Canis lupus pallipes</i>	m	blood	Bhal, Gujarat outskirts of Velavadar National park	IW-A	AY333743	02-Jun-96	Y. V. Jhala
D99	<i>Canis lupus pallipes</i>	m	ear pinna	Bhal, Gujarat outskirts of Velavadar National park	IW-A	AY333743	02-Jun-96	Y. V. Jhala

Himalayan wolf samples from Darjeeling Zoo

DTW2	<i>Canis lupus chanco</i>	f	Hair	Darjeeling Zoo; wolves came to zoo from Spiti Valley, Himachal Pradesh	HW-C	AY333740	09-Mar-02	Dinesh K Sharma
DTW6	<i>Canis lupus chanco</i>	m	Hair	Darjeeling Zoo; wolves came to zoo from Spiti Valley, Himachal Pradesh	HW-C	AY333740	09-Mar-02	Dinesh K Sharma
DTW7	<i>Canis lupus chanco</i>	m	Hair	Darjeeling Zoo; wolves came to zoo from Spiti Valley, Himachal Pradesh	HW-C	AY333740	09-Mar-02	Dinesh K Sharma
DTW8	<i>Canis lupus chanco</i>	f	Hair	Darjeeling Zoo; wolves came to zoo from Spiti Valley, Himachal Pradesh	HW-C	AY333740	09-Mar-02	Dinesh K Sharma
DTW9	<i>Canis lupus chanco</i>	m	Hair	Darjeeling Zoo; wolves came to zoo from Spiti Valley, Himachal Pradesh	HW-C	AY333740	09-Mar-02	Dinesh K Sharma
DTW13	<i>Canis lupus chanco</i>	f	Hair	Darjeeling Zoo; wolves came to zoo from Spiti Valley, Himachal Pradesh	HW-C	AY333740	09-Mar-02	Dinesh K Sharma
DTW15	<i>Canis lupus chanco</i>	m	Hair	Darjeeling Zoo; wolves came to zoo from Spiti Valley, Himachal Pradesh	HW-C	AY333740	09-Mar-02	Dinesh K Sharma
DTW17	<i>Canis lupus chanco</i>	m	Hair	Darjeeling Zoo; wolves came to zoo from Spiti Valley, Himachal Pradesh	HW-C	AY333740	09-Mar-02	Dinesh K Sharma

note: kin relationships among these wolves and the number of founders is uncertain.

Kufari wolf

D192	<i>Canis lupus chanco</i>	f	Hair	Kufari Zoo; animal came from Spiti valley, Himachal Pradesh	HW-C	AY333740	12-May-01	Dinesh K Sharma/YV Jhala
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Wolf samples obtained from Western museum specimens

BM35.1.10.1	<i>Canis lupus "farriger"</i>	m	turbinals	Nepal; "no doubt living animal from Tibet or Kashmir for the Maharajah's collection -RIP"	HW-A	AY333738	29-Jan-17	Reed 21.5.12
BM58.6.24.61	<i>Canis lupus</i>	m	turbinals	Nepal	HW-A	AY333738	?	B. Hodgson
BM99.12.29.1	<i>Canis lupus "farriger"</i>	unk	piece broken skull	Tibet	HW-B	AY333739	?	A.H. Savage-Landor, "skin black" small animal
BM59.670a	<i>Canis lupus (?) chanco</i>	unk	piece broken bulla	Khumbu, E. Nepal	HW-C	AY333740	08-May-09	Zoological Society "Daily Mail" Himalaya Expedition
FMNH 92835	<i>Canis lupus chanco</i>	unk	piece broken skull	Roivaling Valley, Nepal	HW-C	AY333740	14-May-09	E. Hillary et al., fall-winter 1960
USNM 198457	<i>Canis lupus chanco</i>	m	turbinals	Kashmir, Ladakh	HW-D	AY333741	08-Jun-13	W. L. Abbott
USNM 198460	<i>Canis lupus chanco</i>	unk	bone from skin	Tibet, Chan Tung	HW-E	AY333742	1913	via W. L. Abbott

BM35.8.30.2	<i>Canis lupus pallipes</i>	m?	bone	Bikanir, India	IW-A	AY333743	?	"H.H. the heir apparent & pres. of Bikanir"
BM35.1.18.1	<i>Canis lupus pallipes</i>	m	turinals, ligament	Hazaribagh, Dist. Bengal, India	IW-A	AY333743	13-Dec-02	Maj. O. A. Smith, BNHS
USNM 16147a	<i>Canis lupus pallipes</i>	unk	turinals	Karachi ("Kurrachee"), Pakistan	IW-A	AY333743	year 1875	Karachi Municipal Library and Museum
BM56.9.22.24	<i>Canis lupus pallipes</i>	unk	piece broken skull	Punjab	IW-B	AY333744	?	"Lieut. Abbott, 57 rgt."
BM65.1037	<i>Canis lupus chanco</i>	m	turinals	Punjab, Sidhna, near Ravi River, S. tip of Rechna Doab	IW-B	AY333744	26-Nov-64	T. J. Roberts
BM85.8.1.52	<i>Canis lupus pallipes</i>	m	turinals	Sambhar, Rajputana, India	IW-C	AY333745	?	R.M. Adam, prep; A. O. Hume
BM35.1.10.3	<i>Canis lupus "laniger"</i>	f	bone	Rawal Pindi, Upper Punjab, India	IW-D	AY333746	14-Nov-16	Gill. Zool. Soc. rec'd 25 Oct 1912, died 14 Nov 1916.
BM20.1.17.5	<i>Canis lupus "laniger"</i>	unk	ligament bits	Gilgit, Kashmir	WD-W14	AF008141	14-Jun-02	Maj. A.D. MacPherson, "BNHS" "supposed cross with dog"
BM99.6.23.2	<i>Canis lupus "laniger"</i>	unk	turinals, mandible	Braldu, Scardo, Baltistan, Kashmir	WD-W15	AF008142	?	purch'd E. Gerrard
USNM 198458	<i>Canis lupus chanco</i>	f	turinals/cartilage	Nubra Valley, Tiggur, Kashmir	WD-W15	AF008142	08-Aug-13	W. L. Abbott -8170
BM33.7.20.6	<i>Canis lupus "laniger"</i>	m	bone	Gilgit, Kashmir; 36N 74E, 7000'; "caught as a cub & known to be about 6 mo. old"	WD-A	AY333747	?	"Major W. T. R. Trevelyan"
USNM 198463	<i>Canis lupus chanco</i>	unk	turinals	Pakistan, Baltistan, Shigar	WD-W15	AF008142	1913	W. L. Abbott
USNM 198462	<i>Canis lupus chanco</i>	unk	bone from skin	Tibet, but possibly Ladakh	WD-W14	AF008141	1913	via W. L. Abbott

BM=The Natural History Museum, South Kensington, UK; USNM=National Museum of Natural History, Smithsonian Institution, Washington, DC, USA; FMNH=Field Museum of Natural History, Chicago, IL, USA.

Dog samples obtained from India

D1P	dog	unk	Hair	Adjoining areas of Pakhui wildlife sanctuary in Tipi region of Arunachal Pradesh (Twang- local breed of dogs)	ID-H	AY333734		Nita Shah
D2P	dog	unk	Hair	Adjoining areas of Pakhui wildlife sanctuary in Tipi region of Arunachal Pradesh (Twang- local breed of dogs)	ID-G	AY333733		Nita Shah
D3P	dog	f	Hair	Adjoining areas of Pakhui wildlife sanctuary in Tipi region of Arunachal Pradesh (Twang- local breed of dogs)	ID-I	AY333735		Nita Shah
D4P	dog	unk	Blood	Outskirts of Velavadar National Park	ID-I	AY333735		Y.V. Jhala
D6P	dog	f	Hair	Adjoining areas of Pakhui wildlife sanctuary in Tipi region of Arunachal Pradesh (Twang- local breed of dogs)	ID-G	AY333733		Nita Shah
D6P	dog	m	Hair	Adjoining areas of Pakhui wildlife sanctuary in Tipi region of Arunachal Pradesh (Twang- local breed of dogs)	ID-G	AY333733		Nita Shah
D7P	dog	f	Hair	Outskirts of Velavadar National Park	ID-I	AY333735		Y.V. Jhala
D8P	dog	m	Hair	Bhutia Dog from Palampur Valley, Himachal Pradesh	ID-J	AY333736		Dinesh k. Sharma
D9P	dog	m	Hair	Bhutia dog from Kullu valley, Himachal Pradesh	ID-H	AY333734		Dinesh k. Sharma
D10P	dog	m	Hair	Bhutia dog from Kangra Valley, Himachal Pradesh	ID-H	AY333734		Dinesh k. Sharma
D11P	dog	m	Hair	Bhutia dog of nomadic Gaddi (shepherders) tribe of Himachal from Chamba	ID-I	AY333735		Dinesh k. Sharma
D12P	dog	m	Hair	Bhutia dogs from Upper reaches of Chama district Himachal Pradesh	ID-A	AY333727		Dinesh k. Sharma
D14P	dog	m	Hair	Bhutia dog from Upper reaches of Kullu valley	ID-H	AY333734		Dinesh k. Sharma
D15P	dog	m	Blood	Feral dog from Outskirts of Velavadar National Park	ID-I	AY333735		Dinesh k. Sharma
D16P	dog	unk	Hair	Feral dog from Kachchh, Abdasa Taluka	ID-J	AY333736		Y.V. Jhala
D17P	dog	unk	Hair	Adjoining areas of Pakhui wildlife sanctuary in Tipi region of Arunachal Pradesh (Twang- local breed of dogs)	ID-K	AY333737		Y.V. Jhala

D18P	dog	f	Blood	Feral dog from outskirts of Velavadar National Park	ID-I	AY333735	Y.V. Jhala
D19P	dog	m	Hair	Adjoining areas of Pakhui wildlife sanctuary in Tipi region of Arunachal Pradesh (Twang- local breed of dogs)	ID-E	AY333731	Nita Shah
D20P	dog	m	Hair	Adjoining areas of Pakhui wildlife sanctuary in Tipi region of Arunachal Pradesh (Twang- local breed of dogs)	ID-F	AY333732	Nita Shah
D21P	dog	m	Blood	Feral Dog from Outskirts of Velavadar National Park	ID-C	AY333729	Y.V. Jhala
D22P	dog	m	Hair	Adjoining areas of Pakhui wildlife sanctuary in Tipi region of Arunachal Pradesh (Twang- local breed of dogs)	ID-B	AY333728	Nita Shah
D23P	dog	m	Blood	Velavadar National Park	ID-D	AY333730	Y.V. Jhala
D24P	dog	unk	Tissue	Feral dog from Abdasa district of Kachchh	ID-J	AY333736	Wolf research team
W184	wolf dog hyb (captive)	m	Blood	Surendranagar, Gujarat	ID-K	AY333737	