Genetic insights to assist management of the Critically Endangered hangul *Cervus hanglu hanglu* in the Kashmir Himalaya

SNEHA NARAYAN, TANUSHREE SRIVASTAVA, GAYATHRI SREEDHARAN, BAPIN K. PANDA, JAVAID HAMEED, KARTHIKEYAN VASUDEVAN and P. ANURADHA REDDY

SUPPLEMENTARY FIG. 1 Alignment of mitochondrial cytochrome b gene partial region (472 bp) of hangul and several other ungulate species. Arrows indicate hangul-specific variations at positions 711 and 784 of mitochondrial cytochrome b gene that were used for designing primers for hangul-specific diagnostic PCR assay.

SUPPLEMENTARY MATERIAL 1 Detailed methodology for species, individual and sex identification.

Species identification - PCR mix of 10µl consisted of 0.75U Taq polymerase (TaKaRa Ex Taq Hot Start Version, TaKaRa, Japan), 250µM of each dNTP, 1X buffer (TaKaRa, Japan), 10X BSA (New England Biolabs, USA), 3pM of each primer and 20ng template DNA. PCR reactions were carried out in an ABI Veriti Thermocycler (Applied Biosystems, USA) with initial denaturation at 95ºC for 10mins, and 40 cycles of denaturation at 95ºC for 30secs, annealing at 54ºC for 45secs, extension at 72ºC for 30secs, followed by a final extension for 7mins at 72ºC. All reactions were set up in duplicates in a hood cleaned with bleach and alcohol, and irradiated with UV light to eliminate PCR contaminants. Negative controls were included in all PCR reactions. Post amplification, PCR products were visualized on 3% agarose gel to detect a 112bp amplicon confirming the presence of hangul DNA.

Individual identification – Reaction mix of 15µl comprised of 1U Taq polymerase (TaKaRa Ex Taq Hot Start Version, TaKaRa, Japan), 250µM dNTPs, 1X buffer (TaKaRa, Japan), 1.25mM $MgCl_2$, 1X BSA (New England Biolabs, USA), 4pM of each primer and 20ng template DNA. Forward primers were fluorescently labelled with HEX at the 5' end. PCR conditions for amplifying microsatellite loci were similar as those described above for species identification except for the annealing temperature which varied for all loci and ranged from 48ºC to 61ºC. Reaction success was checked electrophoretically in 2% agarose gel. Samples which amplified in at least one out of every three reactions were subjected in triplicate to capillary electrophoresis in ABI 3730 Genetic Analyser (Applied Biosystems, USA) along with Genescan LIZ 500 size standard (Thermofisher, USA), and alleles were sized with GeneMapper 5.0 (Applied Biosystems, USA).

Sex identification - PCR reaction mix of 10µl consisted of 0.5U Taq polymerase (TaKaRa Ex Taq Hot Start Version, TaKaRa, Japan), 250µM of each dNTP, 1X buffer (TaKaRa, Japan), 0.75mM MgCl2, 10X BSA (New England Biolabs, USA), 3pM of each primer and 20ng template DNA. A touchdown PCR reaction was carried out in an ABI Veriti Thermocycler (Applied Biosystems, USA) with initial denaturation at 95ºC for 5 mins. The first 12 cycles had denaturation at 95ºC for 30 secs, followed by a drop in annealing temperature after every two cycles by 2ºC from 68ºC to 58ºC for 45 secs, and an extension at 72ºC for 30 secs. This was followed by 28 cycles with annealing temperature at 58ºC, and a final extension for 7 mins at 72ºC.

SUPPLEMENTARY TABLE 1 This as a CSV file available at doi.org/10.1017/S0030605323001266.

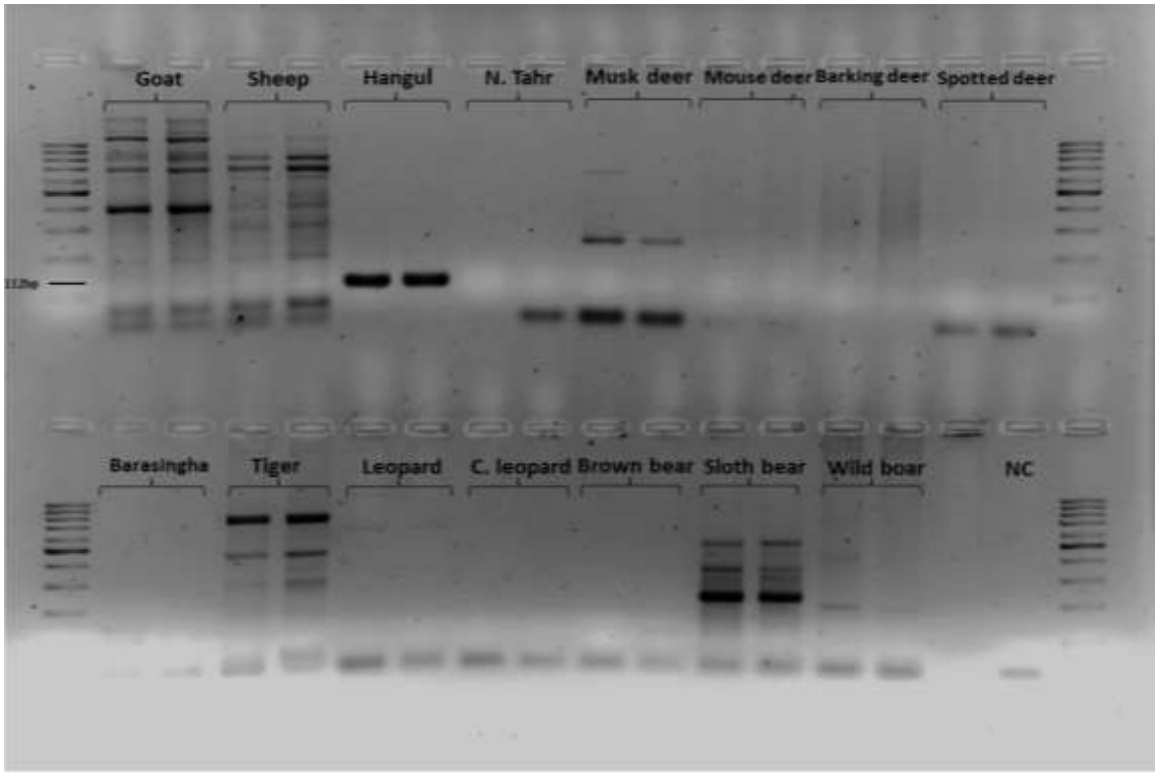
SUPPLEMENTARY MATERIAL 2 Population estimation in MARK.

We arranged the encounter data for analysis using the Robust Design framework in Program MARK. The parameters estimated this way are robust to heterogeneity in individual capture probability. We assumed seven secondary capture occasions within each of the 4 primary capture occasions/ months of sampling the trails. In the months that we did not visit trails over 7 days, we added dots in the encounter history to mark missed visits. So one of our encounter history for an individual looks like '10000..000000.0000000000000. 1 0'. In this example there were five sampling occasions and two missed surveys in the first month, and an individual was identified just once in the first survey. The 1 and 0 at the end of the encounter history denotes that this individual was a male. Sex of an individual was added as a group variable for this analysis. We generated encounter history data for 293 individuals that were genotyped using DNA from faecal pellets collected from these trails. This data was used as input for analysis using the Huggin's closed capture form of Robust design in Program MARK (Huggins, 1989, 1991; Pollock, 1982). Robust Design models in Program MARK estimate detection probability (p) and recapture probability (c) within the primary periods/ months, the Survival probability (S) between the months of sampling, emigrations (Y") and immigrations (Y') and $\hat{N}$ as a derived parameter, abundance estimate for each primary period/ month. We built models in MARK to estimate:

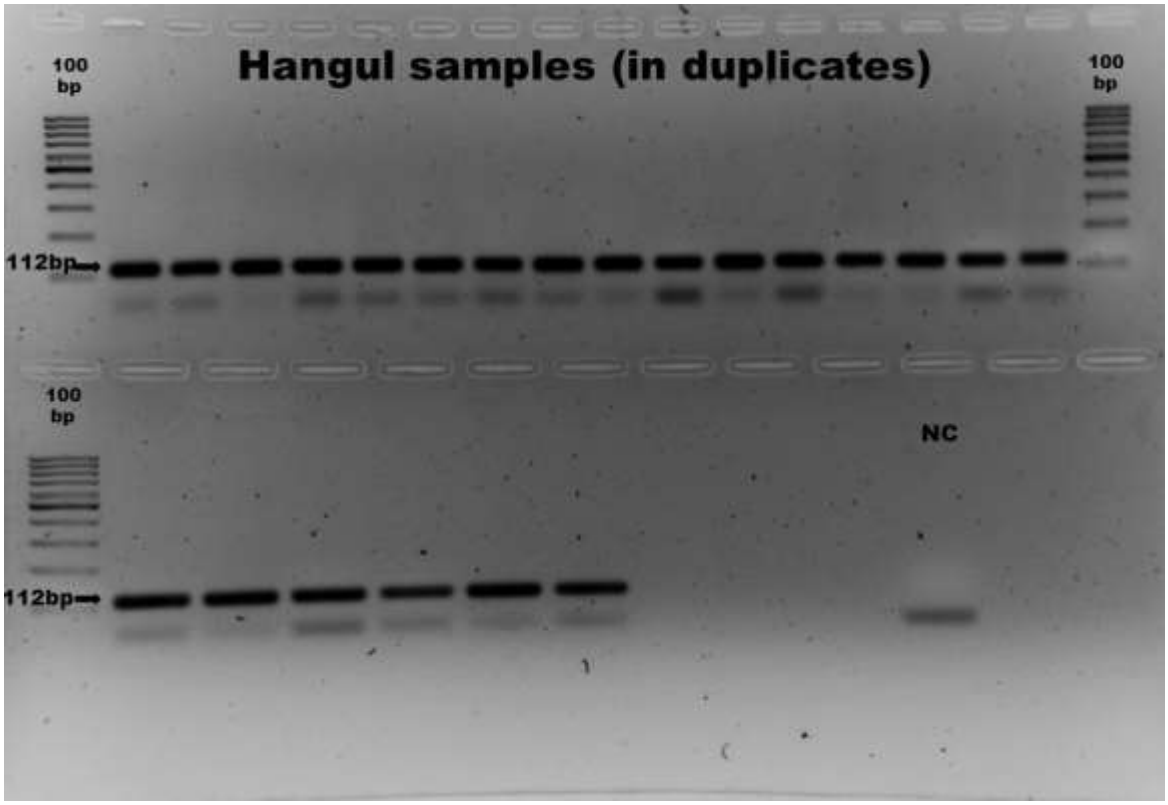
1. detection probability for both the sexes and whether it varies with time of sampling

2. behaviour effect of sexes on capture

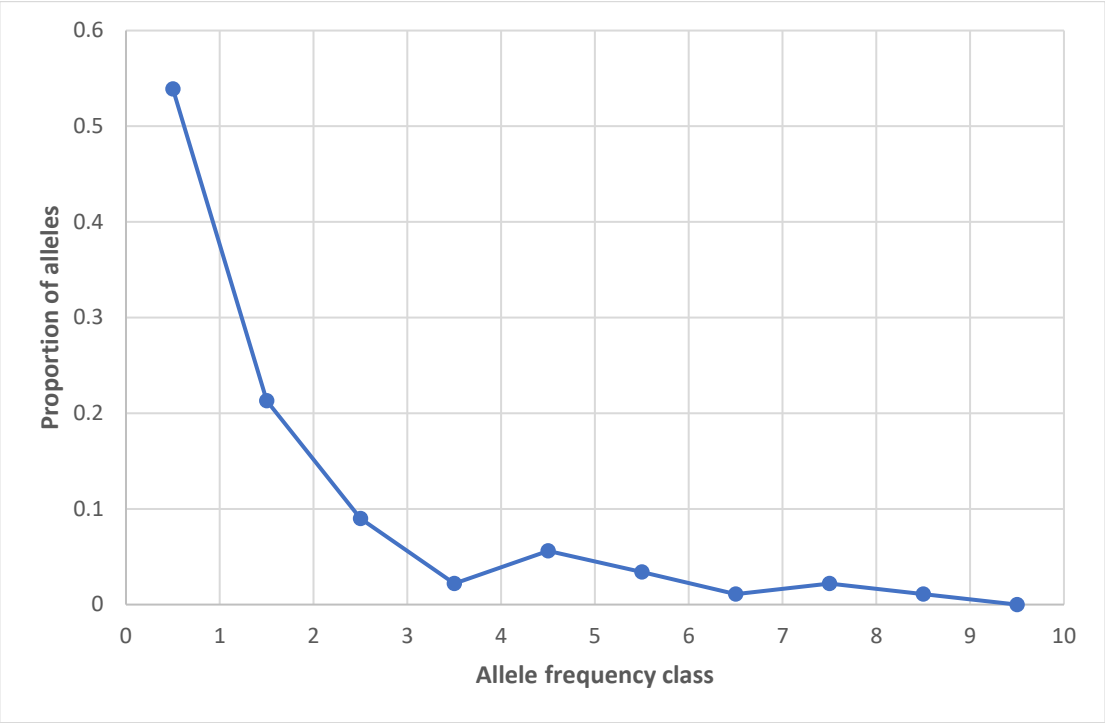
3. population size for all the months and for both the sexes.



SUPPLEMENTARY FIG 2A Hangul-specific PCR assay tested with multiple species. Hangul samples show a 112 bp amplicon not seen in other species.



SUPPLEMENTARY FIG 2B Species confirmation of faecal pellets collected from Dachigam National Park with hangul-specific PCR assay

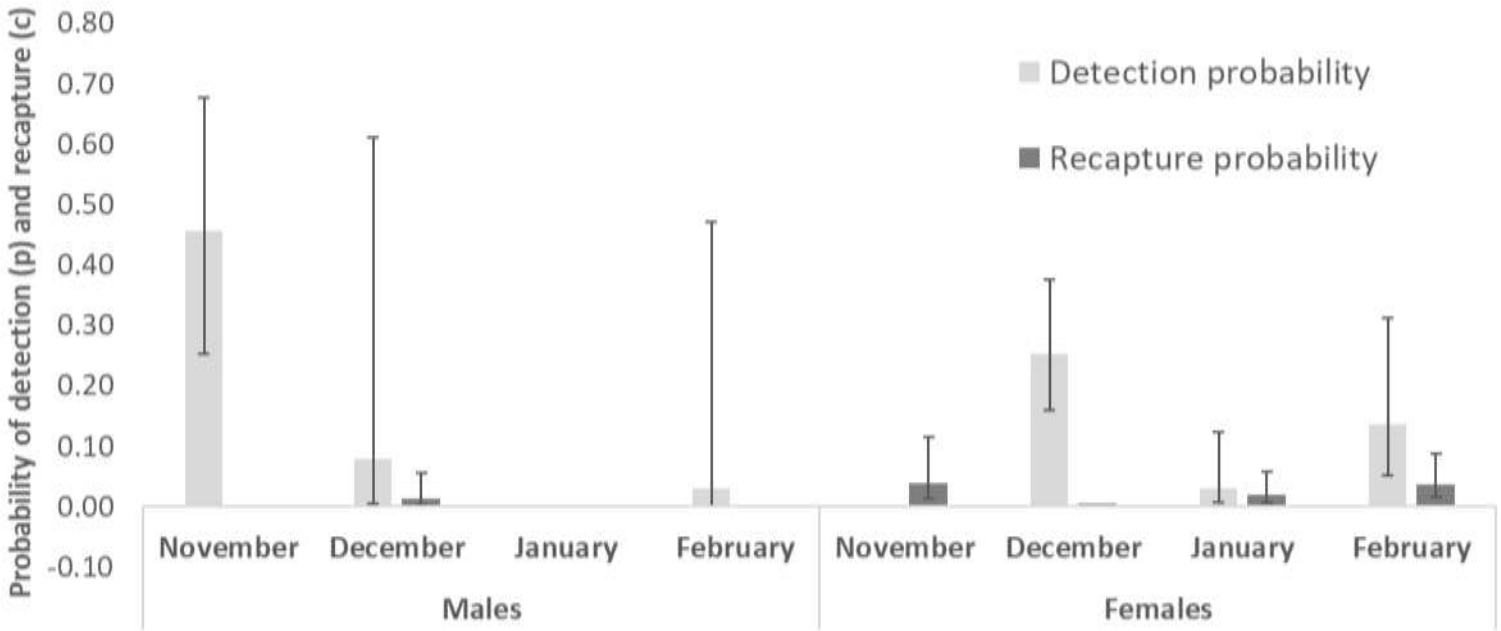


Allele freq class	Proportion of alleles
0.5	0.539
1.5	0.213
2.5	0.09
3.5	0.022
4.5	0.056
5.5	0.034
6.5	0.011
7.5	0.022
8.5	0.011
9.5	0

SUPPLEMENTARY FIG 3 L-shaped mode-shift graph showing a lack of genetic bottleneck in hangul population in Dachigam National Park

SUPPLEMENTARY TABLE 2 Model output from analysis in Program MARK

Models	**AICc**	**Delta AICc**	**AICc Weights**	**Model Likelihood**	**Num. Par**	**Deviance**	**-2log(L)**
S(sex).p(t.sex.)c(t.sex.)	1294.2760	0	0.43729	1	18	1173.860	1255.9730
S(.).p(t.sex.)c(t.sex.)	1294.8971	0.621132	0.32055	0.7330	17	1176.7311	1258.8434
S(sex).p(females.)c(females.).p =c(males)	1297.521	4.8582	0.05203	0.0881	14	1186.013	1268.126
S(sex).p(t).c(t)	1297.842	5.1788	0.04432	0.0751	10	1195.008	1277.12
S(sex).p(t).c(sex.t)	1297.958	5.2951	0.04182	0.0708	14	1186.45	1268.563
S(sex).p(sex.)c(sex.).p =c(females)	1304.116	11.4527	0.00192	0.0033	14	1192.608	1274.72
S(sex).p(sex.)=c(sex.)	1305.293	12.6302	0.00107	0.0018	9	1204.593	1286.705
S(sex).p(t)=c(t)	1309.323	16.6597	0.00014	0.0002	6	1214.938	1297.051
S(.).p(sex.)c(sex.)	13995.47	12702.803	0	0	17	13877.3	13959.41



SUPPLEMENTARY FIG 4 The detection (p) and recapture probabilities (c) of male and female hanguls with 95% CI during the months surveyed in Dachigam National Park.

SUPPLEMENTARY TABLE 3 Model averaged estimates of abundance estimates for males and females across the sampled months.[AQ Some of the numbers here are very large/small. Are they correct?]

	Males			**Females**		
Primary Occasions	**Population Estimates**	**LCI**	**UCI**	**Population Estimates**	**LCI**	**UCI**
November	18	15	20	5482	not estimable	not estimable
December	24681950	-27346285001	27395648901	94	75	114
January	not estimable	not estimable	not estimable	257	-86	600
February	774	-1523.44	3073	76	30	123

References

HUGGINS, R. (1989) On the Statistical Analysis of Capture Experiments. *Biometrika*, 76, 133–140. doi: 10.1007/978-1-4613-8560-8_23

HUGGINS, R.M. (1991) Some Practical Aspects of a Conditional Likelihood Approach to Capture Experiments. *Biometrics*, 47(2), 725. doi: 10.2307/2532158

POLLOCK, K.H. (1982) A capture-recapture design robust to unequal probability of capture. *Journal of Wildlife Management*, 46(3), 753–757.