

## Mitochondrial evidence of phylogenetic distinction of Sri Lankan wild hare (*Lepus nigricollis singhala*)

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### Abstract

*Lepus nigricollis* is a small mammal species mainly found in South Asia. The phylogenetic position of the Sri Lankan wild hare (*Lepus nigricollis singhala*) is yet to be determined. Therefore, the mitochondrial DNA D-loop of three Sri Lankan wild hare samples was studied. Phylogenetic analyses revealed that Sri Lankan wild hare clustered as a basal separate branch consistent with the phylogenetic position of other isolated populations. The limitation of the current data did not permit us to make any further conclusions; therefore, more research evidence is still required.

**Keywords:** D-Loop, *Lepus nigricollis singhala*, phylogeny, Sri Lanka, wild hare

### Introduction

*Lepus nigricollis* is a small mammal species mainly found in South Asian countries like India, Sri Lanka, Nepal, Bangladesh, and Pakistan (Srinivasulu *et al.*, 2004). They generally live in areas with short pieces of bush and small forests that alternate with farmland. *L. nigricollis* is useful for human

and animal consumption, research, sport hunting, and as a pet. The previous study has used epigenetic characters to infer phylogenetic relationships between Indian and Burmese hares (Suchentrunk, 2004). However, the phylogenetic position of the Sri Lankan wild hare (*L. nigricollis singhala*) with others from South and Southeast Asia has not been determined. We herein explored the phylogenetic relationship of Sri Lankan wild hare using mitochondrial DNA (mtDNA) D-loop region comparative to other species.

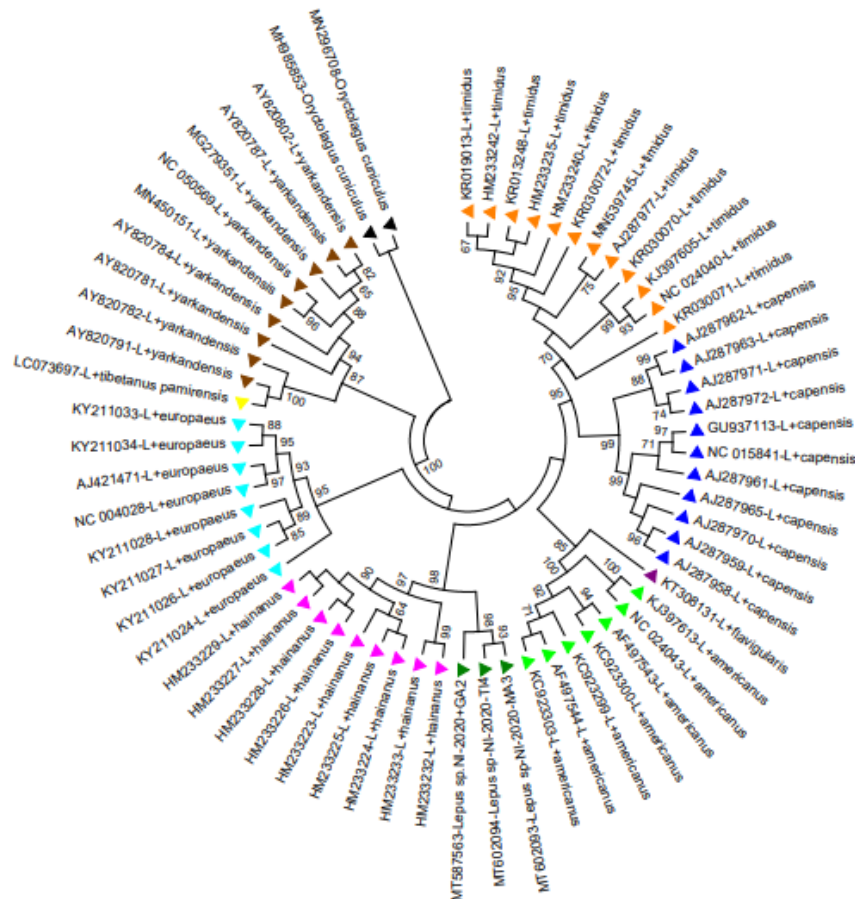
## Material and methods

Tissue samples were collected from three genetically unrelated wild hares from Sri Lanka (Table S1). Genomic DNA was extracted from the tissue samples by the standard phenol/chloroform methods. The mitochondrial DNA D-loop fragment was amplified using the forward primer L15997 5'-CACCATTAGCACCCAAAGCT-3' and reverse primer H16498 5'-CCTGAAGTAGGAACCAGATG-3'. The PCR cycling conditions were 94°C for 3 min, 35 cycles of 94°C for 30 s, 50°C for 40 s, and 72°C for 60 s, and a final extension step of 72°C for 7 min (Fredsted, Wincentz, and Villesen, 2006). The PCR products were purified with ExoSAP-IT as per the manufacturer's instructions (Affymetrix). Sequencing reactions were performed using the BigDye™ Terminator Cycle Sequence Kit 3.1 Ready Reaction Cycle Sequencing Kit (ABI Applied Biosystems). The electropherograms for each of the sequences were visualized, edited, and aligned by SeqMan Pro of DNASTAR Lasergen 7.1.0 (DNASStar Inc., Madison, WI) with the reference sequence KY211026 (Giannoulis *et al.* 2018). A final dataset comprising three Sri Lankan wild hares D-loop sequences (MT587563; MT602093; MT602094) plus 60 rabbit D-loop sequences were retrieved from GenBank (Table S1). Maximum-Likelihood (ML) of the 63 mtDNA D-Loop was constructed using the MEGA version 6.06 (Tamura *et al.*, 2013) with the HKY+I+G model of substitution. The bootstrap test was employed at 1,000 replications to assess the confidence of each node (Felsenstein, 1985). A median-joining network (Bandelt *et al.*, 1999) was also constructed using the program NETWORK 4.2.01.

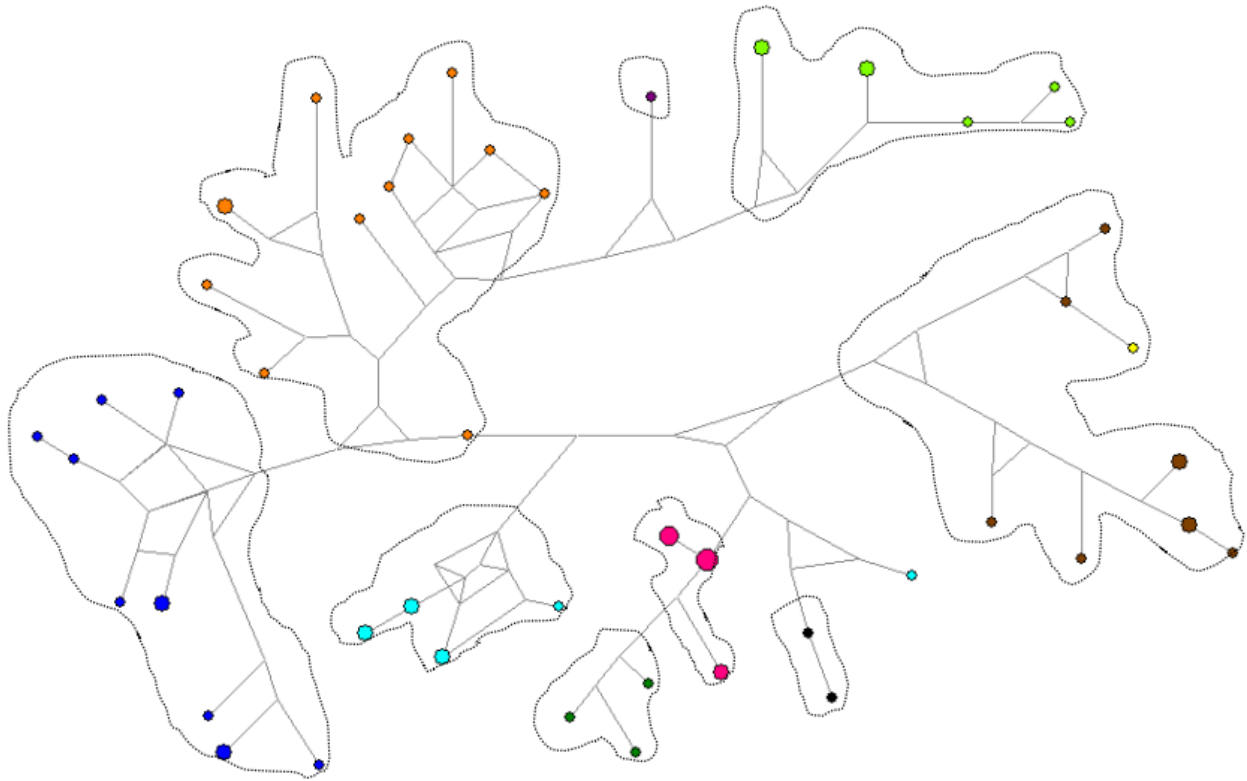
## Results and discussion

Maximum-likelihood (ML) tree of 63 D-loop sequences (Fig. 1; Table S1) revealed that the three Sri Lankan wild hares clustered separately forming a sister clade with *L. hainanus*. Sri Lankan wild hare clustered as a basal separate branch which is consistent with the phylogenetic position of other isolated populations such as *L. hainanus*, only found on the Hainan Island (Kong *et al.* 2016). Hence, the Sri Lankan wild hare individuals are probably a pure ancient hare population in the genus *Lepus*.

Similarly, a network of the 63 D-loop sequences further supports the independent clustering of Sri Lankan wild hare forming a sister clade with *L. hainanus* (Fig. 2). In summary, this study demonstrates that Sri Lankan wild hare is a genetically pure distinct ancient genospecies in the genus *Lepus*. Sri Lankan wild hare cluster as a basal separate branch which is consistent with the phylogenetic position of other isolated populations. The limitation of the current data did not allow us to make any further conclusions; therefore, more research evidence from genetics and even archaeology is still required.



**Figure 1.** A phylogenetic tree of mitochondrial sequences. A phylogenetic tree is based on 63 D-Loop sequences and consists of wild hare from Sri Lanka, America, Africa, Asia, and Europe. All 63 sequences were aligned and trimmed to 498bp. The tree was constructed by the Maximum likelihood method in MEGA version 6.06 (Tamura et al., 2013) with two *Oryctolagus cuniculus* D-Loop sequences (MH985853) and (MN296708) as the outgroups. The numbers indicated at the nodes were bootstraps based on 1,000 replications. Colors indicate the different taxa; Purple (*L. flavigularis*); Lime (*L. americanus*); Blue (*L. capensis*); Pink (*L. hainanus*); Orange (*L. timidus*); Brown (*L. yarkandensis*); Light blue (*L. europaeus*); Yellow (*L. tibetanus*); Green (*L. nigricollis singhala* – Sri Lanka); Black (OG; *Oryctolagus cuniculus*).



**Figure 2.** Median-joining network of mtDNA D-loop haplotypes. Haplotypes are proportional to the total sample number. Colors inside the circles indicate the different taxa; Purple (*L. flavigularis*); Lime (*L. americanus*); Blue (*L. capensis*); Pink (*L. hainanus*); Orange (*L. timidus*); Brown (*L. yarkandensis*); Light blue (*L. europaeus*); Yellow (*L. tibetanus*); Green (*L. nigricollis singhala* – Sri Lanka); Black (OG; *Oryctolagus cuniculus*).

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## References

- Bandelt, H. J., Forster, P., & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular biology and evolution* 16(1), 37-48. DOI: <https://doi.org/10.1093/oxfordjournals.molbev.a026036>.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39(4), 783-791 DOI: <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x>.
- Fredsted, T., Wincentz, T., & Villesen, P. (2006). Introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into wild brown hares (*Lepus europaeus*) in Denmark. *BMC ecology* 6, 17. DOI: <https://doi.org/10.1186/1472-6785-6-17>

- Giannoulis, T., Stamatis, C., Tsipourlianos, A., & Mamuris, Z. (2018). Mitogenomic analysis in European brown hare (*Lepus europaeus*) proposes genetic and functional differentiation between the distinct lineages. Mitochondrial DNA. Part A, DNA mapping, sequencing, and analysis 29(3), 353-360.
- Kong, L., Wang, W., Cong, H., Son Nguyen, T., Yang, Q., Wu, Y., & Li, Y. (2016). Molecular evidence revealed *Lepus hainanus* and *L. peguensis* have a conspecific relationship. Mitochondrial DNA. Part A, DNA mapping, sequencing, and analysis 27(1), 265-269. DOI: <https://doi.org/10.3109/19401736.2014.888550>
- Srinivasulu, C. *et al.* (2004) 'Checklist of lagomorphs (Mammalia: Lagomorpha) of South Asia', Zoos' Print Journal 19(2), 1375-1380. DOI: <https://doi.org/10.11609/jott.zpj.19.2.1375-80>.
- Suchentrunk, F. (2004). Phylogenetic relationships between Indian and Burmese hares (*L. nigricollis* and *L. peguensis*) inferred from epigenetic dental characters. Mammalian Biology 69, 28-45. <https://doi.org/10.1078/1616-5047-114>
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular biology and evolution 30(12), 2725-2729. DOI: <https://doi.org/10.1093/molbev/mst197>.